Micropropagation of juvenile and mature Hevea brasiliensis

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Micropropagation of Juvenile and Mature

Hevea brasiliensis

submitted by

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To my husband

Mahesh

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Abstract

With the aim of establishing a continuous long term micropropagation system for *Hevea brasiliensis*, experiments were carried out with shoot tips and nodes of seedling and rootstock origin (juvenile material) and with similar explants derived from selected clones (mature stock).

When this work was started in 1988, no published protocol existed for the micropropagation of *Hevea* even for juvenile plant material; this problem was therefore addressed first. Contrary to expectations, nodes were found superior as explants compared with shoot tips. The medium suggested by Lloyd and McCown (1980) was found to be superior to that of Murashige and Skoog (1962). As regards plant growth regulators, thidiazuron proved more useful than a mixture of BAP and kinetin. By combining these findings, the outline of a protocol for the continuous micropropagation of juvenile *Hevea* material has been produced.

Explants of mature origin posed problems both of establishment in culture and of reluctance to proliferate *in vitro*. The first problem was overcome by substituting mercuric chloride for sodium hypoclorite as a sterilant and by the incorporation of polyvinyl polypyrrolidon into the culture medium. The second problem has been solved only partially. While primary nodal explants have been induced to expand in culture and buds have been made to proliferate on thidiazuron containing media, no plant growth regulator combination has been found to date which would cause expansion of secondary nodal explants.

Adventitious shoot regeneration has also been attempted using cotyledons, stem pieces, roots and unexpanded leaf lobes of seedlings, but only callus and roots were produced.

With view to develop a procedure for the early identification of *Hevea* plants with a high rubber producing potential, experiments were carried out with stem pieces to measure the extent of incorporation of the radioactive precursors ^{14}C-acetate and ^{14}C-mevalonate into rubber. The uptake of acetate was found to be twice as high as that of mevalonate under the experimental conditions used. Large variation was observed between the percent ^{14}C-acetate incorporated into rubber by stem sections from different individuals within the same clone. Apart from random variation, these differences were presumably attributable to scion rootstock interaction. Stem pieces taken at the resting stage of a growth flush ('mature tissues') were more efficient than those taken at an early stage ('young tissue'); this difference was shown to be owing mainly to differences in dry matter content. Ranking the five experimental clones by ^{14}C-acetate incorporation put the five experimental clones into the same order as ranking them by field rubber yield, with only one exception. The method looks worthy of further study.

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2,4-D	2,4 Dichlorophenoxy acetic acid
IAA	Indol-3-acetic acid
NAA	Naphthalene acetic acid
IBA	Indol-3-butyric acid
GA3	Gibberillic acid
BAP	6-Benzylaminopurine (N^6 Benzyladenine)
Kinetin	6-Furfuryl aminopurine
Thidiazuron	N phenyl 1,2 3-thidiazol-5-ylurea
2iP	(2-Isopentenyl)-adenine
PVP	Polyvinyl polypyrrolidon
M&S	Murashige and Skoog
WPM	Woody Plant Medium
DMSO	Dimethyl sulphoxide
DPM	Decompositions Per Minute
NMR	Nuclear Magnetic Resonance
IPP	Isopentenyl pyrophosphate

Chapter. 1
Introduction

1.1. HEVEA BRASILIENSIS

1.1.1. History and Origin

Successful transportation of a few thousands of seeds from South America to the eastern hemisphere of the world by Henry A. Wickham in 1876 , highlights the historical story of domestication of *Hevea brasiliensis (Wild ex Adr de Jussieu) Muell-Arg*, commonly known as the Brazilian rubber tree or the para rubber tree. Wickham had not known it then, but it is now known,that *Hevea brasiliensis* is the only *Hevea* species indigenous to the Tapajoz in Brazil,where he collected the seeds (Imle,1978; Schultes,1977) and it turned out to be the most important species economically.

In the genus *Hevea*, there are eight more species; *H.nitida*, *H.guinensis*, *H.benthamiana*, *H.pauciflora*,*H.camporum*, *H.spruciana*, *H.rigidifolia* and *H.microphylla*. All are significantly lower yielding than *Hevea brasiliensis* ;the latter accounts for all the natural rubber production in the world today (Chen,1984 ; Schultes, 1970).

1.1.2. Distribution and Production

At present more than 90% of the natural rubber is produced in South East Asia, mainly in Malaysia, Indonesia, Thailand, China, India, and Sri Lanka, in descending order of quantities produced (International Rubber Study Group,1988 ; Rahaman et al,1981). The remainder is produced in Africa and other Asian and Latin American countries,including Liberia, Nigeria, Cameroon, Ivory Coast, Vietnam, Burma, Kampuchea, Bangladesh, Phillipines, Papua-New-Guinea, Singapore, Brazil and Mexico (Figure 1). Attempts have been made to reintroduce selected varieties of *Hevea brasiliensis* to the Western hemisphere, but it was a failure, because all of the genetically improved material was highly susceptible to South American Leaf Blight, caused by the fungus *Microcyclus ulei* (Chee and Wastie,1980 ; Imle,1978).

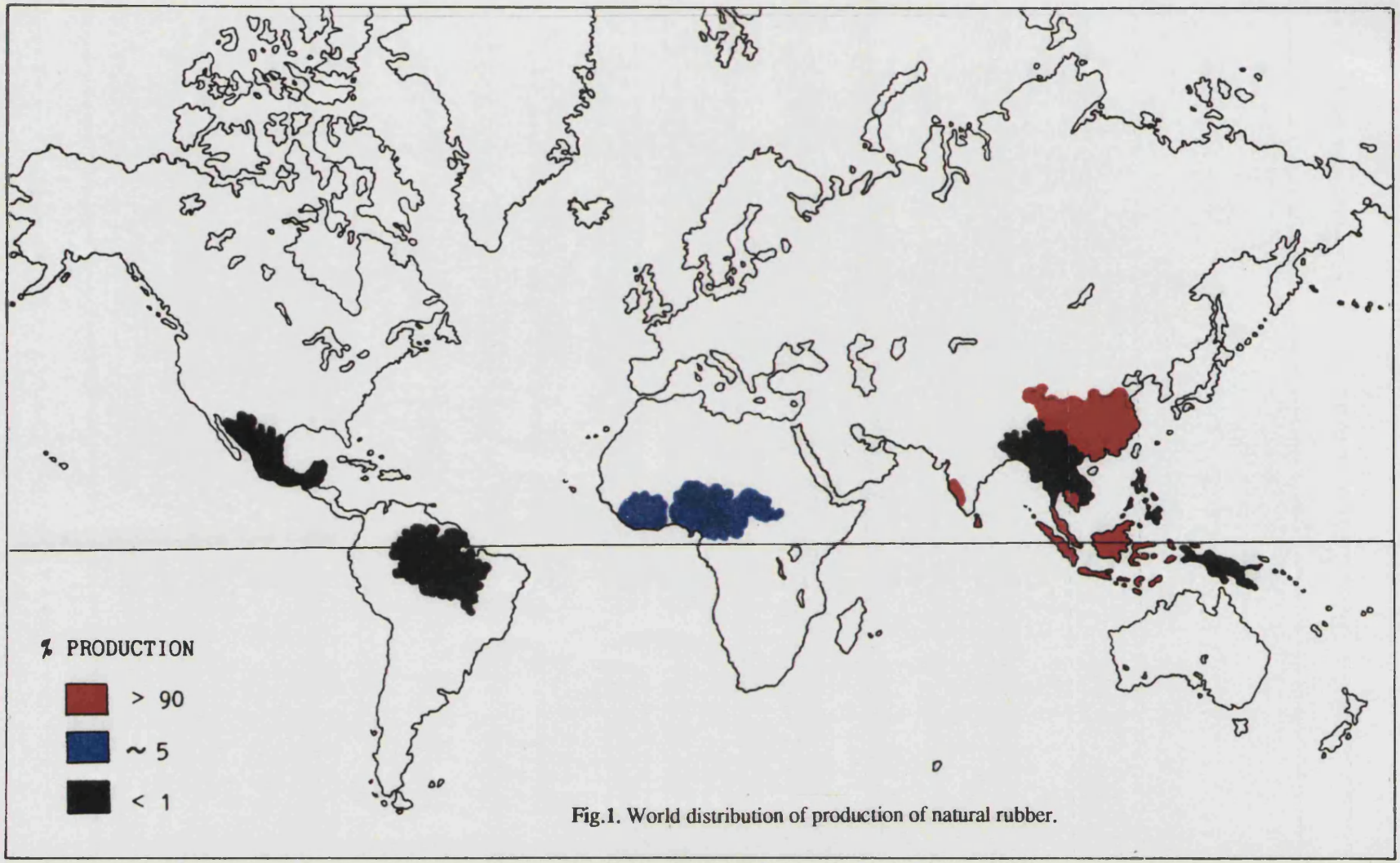


Fig.1. World distribution of production of natural rubber.

1.1.3. Morphology and Taxonomy

Hevea brasiliensis is a highly heterozygous, open pollinated, perennial tree, belonging to the family Euphorbiacea. It is cultivated mostly in high rainfall tropical areas within 20° N and 20° S of the equater. The taxonomy of *Hevea* has been studied by many workers, since its introduction into cultivation (Schultes, 1970). The growth during the first 3-5 years is purely vegetative and after that the bark can be exploited at periodic intervals by removal of a thin shaving. Under favourable conditions, the normal growth curve of a free growing rubber tree, is S-shaped, both for seedlings and for buddings. With buddings during the first three years, after planting in the field as budded stumps, the scions grow upwards without branching.The increase in girth during this period is relatively small,but as soon as terminal growth stops,and the branches begin to develop , girth development of the stem is greatly accelerated. The average stem girth of the trees in plantations is about 100 cm, whereas the Brazilian *Hevea* in the rain forests has an average girth of about 250 cm and a height of over 30 m. Girth development gradually becomes slower after 8-10 years. The same picture is obtained with seedlings but, the stem at the base of the trees soon becomes conical and the shape of the trunk gets tapering, in contrast to the stem of buddings.

It has been found, that there are many physiological changes, occurring in rubber even during its vegetative phase. This has been observed in as early as 1939 (Baptist ,1939).The position on the parent plant from which cutting are taken,has been found to be an important factor for successful rooting. One way of showing the difference between juvenile and adult state of a plant is the capacity to produce roots;thus, in *Hevea* the cuttings from juvenile or young trees regenerate roots readily but cuttings from old trees do not root at all (Muzik,1953; 1956 ; Muzik and Cruzado,1956 ; 1958). This has been described by McIndoe (1958), showing that, the buds which are removed from the stems at heights not more than 3 feet above the root collar, reproduce their seedling characters fully, while at higher levels all buds produce buddings showing the nearly cylindrical shape of the normal budded tree.

1.1.4 Exploitation and Improvement

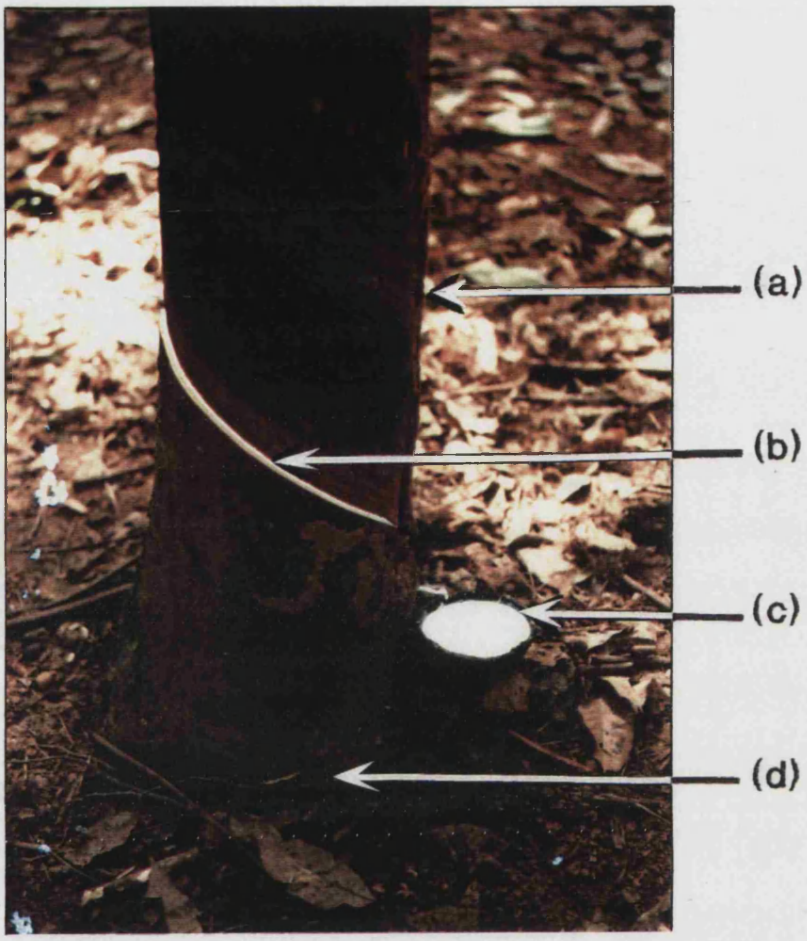


Plate 1. About nine year old rubber tree, under tapping, (a).Tapping pannel, (b).Tapping cut, (c).Latex collected into a coconut shell and (d).Graft union

Although it is considered that the most critical step of commercial plantations of *Hevea* was, the transporting of materials from South America to the Orient, leaving the South American Leaf Blight behind, it was an equally important step to develop a superior tapping system by H.N.Ridley in 1889. His tapping system which caused minimal tree damage,allowed a tree to be tapped 100 or more times per year, and greatly increased the annual yields. With minor modifications, his tapping method remains in use even today (Dickman, 1951; Imle, 1978). The highest yield obtained in the first unselected trees of Wickham, was less than 225 kg per

hectare per year, even with the best tapping method practised then. As a result of selective breeding programmes, the yield has been increased remarkably by more than 10 fold (Chen,1984 ; Paranjothi,1987). But all these high yielding clones originated from Wickham collection.

Although the yield is the main problem addressed by breeding programmes, it is also important to produce strains with characters such as, disease resistance, wind resistance and greater growth vigour.

1.1.5 Propagation

In earlier times, propagation was done by harvesting seeds from selected superior trees grown in special gardens. Vegetative propagation would have been more desirable since, planting of unselected seedlings, even though, originating from seed gardens, results in uneconomic holdings.

Propagation by stem cuttings has not been recommended since, the adventitious root system lacks a tap root,which is very important for a substantial anchorage, as the plant is fully grown. Rooting is not too easy in any case; even a fibrous root system develops only on placed in beds, under a permanent fine spray of water.

Bud grafting of *Hevea* developed in Java by Dutch horticulturists in 1950's, was a real breakthrough in the improvement of plantations of rubber. This made the propagation for commercial plantings of high yielding clonal trees possible. Bud grafting involves the planting of unselected seedlings for the root system, and grafting the desirable clonal bud onto it. Throughout the years, improvements in bud grafting have been made, including its applications to very young seedling rootstocks, and to three component trees. The latter is a tree consisting of a seedling rootstock, a high yielding trunk clone, and a disease resistant canopy clone, united into a single plant through bud grafting. These three part trees, which have been considered a good achievement in the rubber industry, have the advantage of using clones resistant to leaf diseases, such as South American Leaf Blight, for the canopy.

The present method of vegetative propagation of rubber is bud grafting, but, this is an intensive operation requiring skilled labour. Also, the effect of the root stock from the unselected seedling,on the yield of the scion is a problem, which is still unsolved.

The ability to produce roots in cuttings of young *Hevea* plants, has been described in terms of the juvenility of the plant; and the inability to produce roots in mature cuttings, as the losing of juvenile characters. Attempts have been made to reintroduce juvenile characters into mature plants and, thereby enhance the rooting ability by grafting buds from mature plants onto seedling plants (Figure 2).

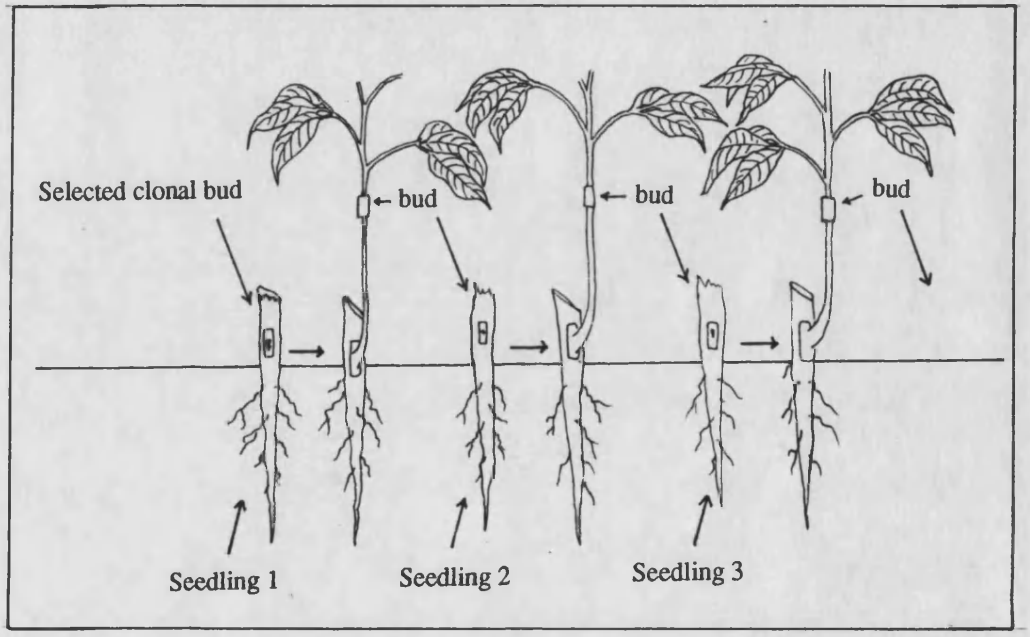


Fig 2. Sequence of repeated bud grafting, used in re-introducing juvenile characters in mature trees.

Repeated sequence of budding, and planting cuttings from them under a mist spray has been done four times, and it has been observed, that 30% of cuttings from the scions of the fourth and fifth graftings, have formed roots in about eight weeks after planting under a mist spray, while

cuttings from the original clone and from the first, second, and third graftings have failed to do so (Muzik and Cruzado,1958).

However, because of the absence of a proper root system on cuttings, and the difficulty or impossibility of inducing rooting in mature cuttings, the requirement of skilled labour for bud grafting, and finally, the unpredictable root-scion interaction involved in budded plants, a proper method of propagation is not available for rubber to produce true-to-type plants.

It would therefore be an advantage to the rubber industry if an alternative method of propagation could be found. *In vitro* propagation shows a promise as a useful technique to help reaching this objective. Attempts at exploiting the tissue culture technique for *Hevea* improvement have already been made, mainly in the rubber growing countries of Asia. Various possible uses of tissue culture techniques for *Hevea* propagation and breeding have been reported by many workers.

In addition to propagation programmes, other avenues of interest of *Hevea* tissue culture include mutation and polyploid induction, haploid plant production and genetic manipulation through the use of protoplasts. The prospects of utilizing these techniques successfully for *Hevea* improvement, will depend on the establishment of well developed tissue culture methods for plantlet production, through shoot tip culture, somatic embryogenesis or organogenesis.

1.2 MICROPROPAGATION OF TREES

'Micropropagation' was originally defined as any aseptic procedure, involving the manipulation of plant organs, tissues or cells that produces a population of plantlets and, enables one to by-pass the normal sexual process or vegetative propagation as conventionally practised.

'Clonal micropropagation' implies that each of the plantlet so produced could grow to be phenotypically and genotypically identical to the original plant from which it was derived.The size of the propagule used in culture is so small, that this *in vitro* asexual propagation technique has been referred to as 'micropropagation'. The use of *in vitro* techniques for asexual propagation is the most advanced area of plant tissue culture.

The success with almost all the techniques of *in vitro* culture, is largely restricted to herbaceous species, and is often sufficiently reliable to be used commercially (Murashige, 1978). Compared to them, achievements with trees have been generally less successful, including propagation *in vitro*. The application of *in vitro* techniques to trees has a relatively short history, although there has been a rapid advancement in recent years. Tissue culture work of woody species has started as early as 1930's, with cambial tissues to give rise to callus (Mott and Zimmerman, 1981). Even though trees are among the first to produce callus cultures, it was not until about 1975, that the rudimentary demonstration of micropropagation, controlled adventitious bud initiation, and somatic embryogenesis were made for trees. *In vitro* shoot regeneration has been achieved in 1950 from cultured burls from the base of *Sequoia* but not from other more mature parts of the tree (Ball,1950).

However, it was not until 1970, that the first plantlets were produced from callus cultures of aspen. In 1975 a micropropagation scheme for *Eucalyptus*, had been put forward and also adventitious buds had been derived in cotyledons of longleaf pine.

Somatic embryogenesis in culture has been reported in *Thuja orientalis* in 1965, and for citrus from protoplasts by 1975. A somatic embryogenesis system for coffee callus has been developed and refined during this period as well (Mott and Zimmerman,1981).

A major feature in the tissue culture of trees is the adaptation of methods demonstrated together with juvenile material to explants derived from more mature stages of growth, which are less tractable in tissue culture systems.

Almond in particular is very difficult to root by cuttings *in vivo* both in the juvenile and the mature stages, although many woody species are difficult to root only after the juvenile stage (Kester and Sartori, 1965). In an attempt to develop an alternative to grafting of almonds, rapid shoot proliferation has been obtained with a multiplication rate of about 6 shoots in 20 days. Rooting was induced in more than half of the shoots, in the presence of NAA. Improvement in the rooting percentage (more than 70%) was noted with prolonged subculturing. In the field, surviving plantlets developed a good root system (Rugini and Verma, 1982).

Mature elite trees of teak, *Eucalyptus*, tamarind and pomagranate have successfully micropropagated and established in the field (Mascarenhas et al, 1981). Although adult *Eucalyptus* trees are difficult to propagate by conventional methods, superior plants with a high multiplication rate have been obtained by tissue culture techniques. Ten months old micropropagated plants contained more or less the same oil content of that of three years old trees propagated conventionally. Using conventional rooting of cutting techniques *Eucalyptus* can be propagated only from juvenile nodes up to node 14 but was propagated in tissue culture from nodes up to node 50 (Cresswell and Nitsch, 1975). Also, marked increase in rooting ability of nodal explants in culture was reported.

As far as tamarind is concerned, there are no methods available for vegetative propagation by conventional methods. From seed raised plants, flowering occurs only after 15-20 years of germination. Therefore it would be an added advantage if the micropropagated plants flower and fruit early.

Perennial fruit trees can have juvenile periods that last 6-15 years. This together with the genetic complexity of these trees, has meant that conventional plant breeding approches have had relatively little impact on cultivar improvement. Adventive embryony commonly occurs in trees of the tropical rain forests. Many tropical fruit tree species are naturally polyembryonic including *citrus*, mangosteen (*Garcinia mangostana)*, mango (*Mangifera indica*), several species of *Eugenia* or *Syzygium*, langsat (*Lansium domesticum*) and jaboticaba (*Myrciaria cauliflora*). The

germination of somatic embryos of these has been difficult to demonstrate with any consistency except for *citrus* (Litz, 1985).

Tropical Trees and Their Growth

Episodic growth is characteristic of many tropical tree species. It is an important consideration when choosing explants for vegetative propagation, because of the possible physiological and hormonal changes occuring in the plant, in connection with their vegetative growth pattern.

Factors influencing flushing are varied and complicated. In flushing, an individual shoot passes through alternate phases of growth and dormancy. With each growth phase or flush a variable number of leaves expand and the adjacent internodes elongate. The terminal bud enters a state of dormancy until the next growth period begins.

This type of shoot growth in tropical trees has been discussed in relation to various factors such as temperature (Sale,1968), with many tropical trees such as *cacao* ((Sale, 1968), tea (Bond,1945), mango (Greathouse et al,1971), macadamia (Stephenson and Cull,1986) and *Oreopanax* (Greathouse et al, 1971).

In many of these studies it was found that growth periodicity can be attributed to internal factors that are independent of the environment. Evidence from *Theobroma cacao* has supported the notion that the basic controlling factors for growth and dormancy cycles in tropical trees are endogenous (Greathous et al, 1971).

Other workers (Sale, 1968, Stephenson and Cull, 1986) suggested that factors influencing flushing are mainly environmental such as temperature, rainfall and soil nutrition. In the case of

macadamia, threshhold temperature for flushing behaviuor in the field is 10-30 C (Stephenson and Cull, 1986) and flushing peaks occur in late summer and early spring, although smaller flushes sometimes occur at other times.

It has also been found that the growth rhythum in tropical trees is not easily correlated with environmental factors except in reions subject to seasonal drought. *Theobroma cacao, Mangifera indica, Camellia thea* and *Oreopanax* have supported this theory (Greathoux et al, 1971). Given the environmental conditions favourable for growth, the endogenous rhythum can be expressed and regular phases of growth and the dormancy are the results.

Flushing in *cacao* has been considerably greater at the higher temperatures, partly as a result of the loss of apical dominance (Sale,1968). The flushing behaviour of tea has been analysed in terms of apical activity involving the production and emergence at the apex of new primordia, and the primordial growth rate as affecting the subsequent development of the primordia into foliage (Bond,1945). Although the production of primordiaby the apex can be a continuous process, this varies according to the stage in the flush cycle. Cyclic growth patterns of shoot which can be related to periodic changes in the physiology of the tree have been observed in *Citrus* species. The timing and the rate of growth of vegitative flushes are influenced by climate and soil temperature (Syvertsen et al, 1981).

The growth periods involved in flush growth normally characterized by the expansion of leaves and elongation of the shoot as stated earlier. During dormancy, the length of the shoot remains constant and no new leaves expands. The total number of leaves and leaf primordia in the shoot apex remains constant during the dormant period and does not increase until the onset of the growth period. This indicates that activity of the apical meristem as well as leaf expansion and shoot elongation are rhythime in trees with flush growth pattern. In *Hevea* the growth occurs

in flushes in both seedlings and in grafted mature plants. The length of the shoot in the growth flush decreases with the number of flushes.

From the above, it will be appriciated that the condition of the stock plant prior to initiation into culture is very important. From juvenile or mature trees, shoot tips or nodal explants may be taken prior to, during or after active growth. Any attempt to culture trees exhibiting episodic growth should take into account these differing physiological states. Few workers have directly discussed the problem of episodic growth in relation to selection of explants for initiation into culture. Most reports in the literature are concerned with difficulties of working with mature tissues, and have looked at ways of 'rejuvenating' the stock plants through severe pruning or selection of epicormic shoots (see section 1.2.1 a). The present thesis addresses the problem of explant selection at different points in the flushing cycle because this could have a major impact on the success of the tissue culture system.

Root System of Tropical Trees

Hevea brasiliensis is known to have a tap root system (Muzik, 1953) which provides substantial anchorage when the tree is mature and fully grown. Attempts have been made to propagate *Hevea* by rooted cuttings and it has been found that the twigs of mature trees do not form roots whereas, stem cuttings from the base of seedling plants produce roots. But the trees grown in this way lacked tap roots and the lateral roots developed were usually not strong enough (Dickman,1951) as the tree mature. Proper tap root system also support for the uptake of water and soil nutritions from deep in the soil which helps the tree to grow vigorously.

In cashew (*Anacardium occidentale)*, existance of a dominant tap root in seedlings has been reported (Arglus, 1988). In older trees, the tap roots are said to penetrate very deep into the soil, thus playing an important part in the resistance of the trees to drought conditions.

Consequently, the nature of the root system, macro or micro, could be very important as the tree matures, and any root system should be considerd in this light.

1.2.1 Problems Associated with Micropropagation of Trees

It is clear, that most of the problems encountered in *in vitro* culture are generally common to most trees. Among them, the maturation or the aging of plants, has become an inevitable consequence of the woody perennial habit. Therefore, it has been difficult to demonstrate *in vitro* regeneration pathways for most of the woody perennial plants, because of the apparent loss of juvenility or regenerative potential in mature trees (Bonga, 1982).

Difficulty in sterilization of field grown materials to establish *in vitro* is also a problem, mainly due to the age of them.

By overcoming these problems, however, considerable work has been done successfully to use *in vitro* techniques with woody plants, and,by 1978, commercial production of some woody plants also started (Zimmerman, 1985). Production of rootstocks by means of tissue culture is in commercial scale for both apple and peaches (Jones and Hadlow, 1989).

(a). Physiological State of the Explant

Explant source, is one of the most important practical consideration in the application of tissue culture techniques to trees. To develop such techniques, features such as juvenility, maturation and rejuvenation of plant materials has to be considered.

Juvenility and Maturity

The juvenile and adult phases of the life cycle of woody perennials, and the mechanisms which control the expression of juvenile and adult characters, have been disscused vastly but studied in detail, in relatively a few species (Bonga,1981; 1982; 1987; Borchert, 1976; Durzan,

1984; Fortanier and Jonkers,1976; Franklet et al, 1987; Hackett,1985; 1987; Heybroek and Visser, 1976; Mullins et al, 1979; Pierik, 1990; Robbins,1957; Robinson and Wareing, 1969; Romberger, 1976).

The capacity to vegetatively propagate trees, is associated with juvenility. Generally, the more juvenile the specimen, the easier it is to propagate vegetatively. But, there is no clearly defined transition from the juvenile to the mature phase in most plants. Often, some parts of the tree may be mature or senescent while other parts still display juvenile characteristics. In some trees, major morphological characteristics may change abruptly when maturing, while in others, the changes are more gradual (Bonga, 1982). For vegetative propagation purposes, it is important to be able to recognize which trees, or which tissues or parts of tissues are juvenile.

Maturation can be defined as the developmental process inducing changes in morphological and physiological characteristics leading to the reproductive state. But, it does not seem to be a permanant change in the genome, since it is the adult phase which produces the seeds which in turn give rise to seedlings with juvenile characteristics (Pierik, 1990).

Maturation is strongly correlated with flowering. Accordingly, juvenile phase is often defined as the period where flowering does not occur and cannot be induced by normal flower inducing treatments. Although in this defenition the most general characteristic of juvenility is the inability to flower, maturation is certainly not a single on/off event restricted to flowering, but an accumulation of events in time such as changes in rooting ability and quality, growth rate, plagiotropism, leaf morphology, phylotaxis, anthocyanin content, hairiness, leaf retention, branch angle and thorniness (Pierik, 1990). An important mature characteristic of cells or tissue is that they have lost the capacity for adventitious organ or embryo formation. Meristematic apices, the centers of growth and organization in plants undergo changes when the plant matures. Therefore, the tissues derived from these apices behave differently in young and old plants (Bonga, 1980 , Hackett, 1980). One consequence of this is that shoot cuttings from older trees often root poorly or not at all. Furthermore, if rooting occurs, the propagules may not behave true-to-type, but

show undesired characteristics such as plagiotropic growth, reduced growth rate etc. (Hood and Libby, 1978).

It is belived that juvenility is an unstable state which exists in the meristems and which proceeds through a series of steps to a relatively stable state which is a characteristic of the adult meristem. Changes occur in the buds with time, so that they aquire a certain individuality persisting even after a bud is seperated from the plant. When adult meristems are isolated and/or used in grafting experiments, they remain adult and are not easily changed (Durzan, 1984).

Rejuvenation

In the application of tissue culture to mature trees, obtaining relatively juvenile material from mature plants is very desirable, because they are more easy to handle in tissue culture systems. One of the most common methods is to use juvenile parts of mature plants. Most trees have zones that retain a degree of juvenility longer than other areas of the tree (Romberger, 1976). Roots often retain juvenility and thus a capacity for vegetative propagation and juvenile types of growth for a long time (Norezan et al, 1971). In the above ground part of the tree, the less distance a shoot apical meristem is located from the base of the trunk, the more juvenile it generally is. In a regular shaped tree, like a conifer, this means that the meristem at the apex of the leader is the least juvenile. Also the floral parts posess a high degree of regenerative potential *in vitro*, possibly because of their proximity to the rejuvenating sexual cells (Nozeran et al, 1971).

Sometimes, there are reasons other than vegetative propagation why maintanance or reestablishment of juvenility is of interest. The juvenile phase of tree growth has some atributes which are important in practical forestry. Firstly, when trees mature their growth rates often drop. Secondly, in many hardwoods the trunk is single as long as tree is juvenile but, starts to fork when the tree reaches maturity (Bonga, 1982). Thirdly, juvenile trunks have more radial growth and the juvenile bark is more resistant to desease and stress (Sweet, 1973). However, juvenile

characteristics are not always the ones desired. Fiber length is short in juvenile wood and in conifers the mature growth form is sometimes better than the juvenile one (Sweet, 1973).

Although, there is considerable evidence to indicate that mature meristems are quite stable both *in vitro* and *in vivo*, their phase related characteristics can be modified as a result of *in vitro* culture. The length of the culture and the number of subcultures involved seem to be related to such changes. Shoot production and rooting ability of apple cultivar M.9 increased with subculture (Webster and Jones, 1989). David et al (1978) have reported the *in vitro* rejuvenation of *Pinus pinaster* primary meristems in culture. High concentrations of BAP and low concentrations of sucrose favoured juvenile characteristics. Thornless blackberries cultured from shoot tips, produced monophyllous leaves. Shoot cuttings with this type of leaves are easily rooted which is a juvenile characteristic. The resulting plants with monophyllous leaves produced trifoliate leaves once established *in vivo* (Broome and Zimmerman, 1978).

Some of the juvenile characteristics were induced from the buds of an adult clone of *Vitis vinifera* after 2 to 3 subcultures (Mullins et al, 1979). Rooting percentage increased from 10% in the primary cultures to 60% in the second and subsequent subcultures of the bud cultures of 199-year-old *Tectona grandis* tree (Gupta et al, 1981). Rejuvenation *in vitro* of many apple cultivars and thereby increasing capacity of the shoots to initiate adventitious roots have been reported (Jones, 1983).

The use of explants from naturally rejuvenated tissues has been advantageous with some angiosperm forest trees. Explants from suckers of *Populus* spp began to grow and proliferate on the nutrient medium immediately, whereas those from the aerial shoots frequently did not begin to grow for several months (Christie, 1978).

Multiple grafting of scions from the selected mature trees onto a seedling root stock has been used effectively in some conifers (Cresswell et al,1982 ; Zimmerman, 1985). Once the graft is successful and the scion has grown, the tip of the new shoot is grafted onto another seedling. This procedure is repeated until the scion exhibits juvenile characteristics, or responds like a

juvenile plant. Transmission of rooting ability, which is a juvenile characteristic, from seedlings to adult *Hevea brasiliensis*, using successive bud grafting has been demonstrated (Muzik and Cruzado,1958).

Application of sprays, pastes or injections of synthetic plant growth regulators, has been done by spraying plants with gibberillins or benzylaminopurine (Robbins, 1957). Mature *Hedera helix* can be rejuvenated to varying degrees by treatments with doses of GA_3. The stability of the characteristics induced by GA_3 depends on the dosage (Roglar and Hackett, 1975). Application of GA_3 to mature pear, *Citrus*, *Acacia* and some prunus species inhibited flowering and induced juvenile morphological characteristics (Hackett, 1985).

Severe pruning has been used successfully to obtain shoots with higher rooting potential in apple, *Pinus radiata* and several *Eucalyptus* species (Hackett, 1985, Libby and Hood, 1976). Pruning the stockplants of plum severly in winter enhanced rooting of both the conventional and *in vitro* derived cuttings compared with higher pruning (Howard, et al, 1989). Alteration of the balance of vegetative and reproductive growth through girdling, light treatments, root pruning, and addition of nitrogenous fertilizers, or growth retardants, Preconditioning explants, etc. has also been found to rejuvenate mature species. However, it is evident that the rejuvenation is a prerequisite for possible cloning of adult trees and that the success in practice will mainly depend on the ability to rejuvenate them (Boulay, 1987 , Pierik, 1990).

(b). Phenolic Browning

Brown exudates or phenolics from the explant followed by the death of the explant, is also an important and frequent problem among *in vitro* cultures of trees (Compton and Preece,1986). Various methods of eliminating brown exudates or preventing its formation have been tried, but, rarely with complete success (Cresswell and Nitsch,1975 ; Cresswell and Nitsch,1977). It has been described, that some exudates produced as a result of wounding, appears not immediately

but, some time after the excision of the explant, and is aggravated by certain constituents of the culture medium such as high sucrose, serine, chlorogenic acid and cytokinins, high boron concentration and light. Some other exudates, appearing at the end of the incubation period, seem to be products of dying cells.

It has been found that the damage due to some exudates can be reduced by polyvinylpyrrolidone (Christiansen and Fonnesbech, 1975; Walkey, 1972). Antioxident solutions,such as, ascorbic acid with citric acid have also been found useful for maintaining tissues after initial excision, prior to disinfection (MaComb and Newton, 1981), in reducing phenolic exudates. Incorporating antioxidents into the medium has been successfully tried with some other species (Zimmerman, 1985).Use of activated charcoal, has been tried with success (Preil and Englenhardt,1977 ; Tisserat, 1979 ; Wang and Huang 1976), but the absorption of growth regulators by charcoal is found to be a disadvantage. Transferring explants to fresh media frequently for a period upto several months, has also been successfully used (Anderson,1975 ; Broome and Zimmerman, 1978 ; Lloyd and McCown,1980). Soaking explants in water prior to culture has been an effective method of reducing phenolic browning for some plant species (Rodrigue,1982). Use of low salt media in combination with soaking, has been effective for some other plants (Chevre et al,1983).

(c). Disinfection

One of the major reasons that more work has been done with seedling materials is, that besides the greater plasticity of the juvenile tissues; it is possible to grow seedlings aseptically and, so eliminate the problem of sterilizing field grown tissues.

The surfaces of the plants carry a microbial flora, which, while not being harmful to the plants, multiplies rapidly on the culture medium. When working with field grown plants, specially trees, tissues like stems can be very old, evidently microbes penetrate within the plant tissue, and are extremely difficult to disinfect. This indigenous microbial contamination had been

a serious problem with most of the forest trees (Fossard et al, 1977). To reduce this type of contamination, extending of surface sterilization by soaking the explants overnight in running tap water, or in a weak solution of calcium hypoclorite, prior to the normal surface sterilization, has been tried (Cresswell and Nitsch, 1975). But, the success of this procedure depends on the sensitivity of the tissue to the deconaminant used.

In some instances, contamination problem can be overcome, by growing proliferating cultures under conditions favouring rapid shoot elongation, like low cytokinin medium and/dark, followed by culturing shoot tips from the proliferated shoots. Cultures from such shoot tips will often be found to be free of bacterial contaminants (Zimmerman,1985). Incorporating antibiotics in to the culture medium, has been reported to control bacterial contaminations effectively, in a number of plant genera (Carron et al, 1985; Phillips et al, 1981; Young et al,1984).

Another method which has been tried, is protecting the new growth of shoots. Even in a glass house, by covering with polythene bags, reduced the contamination rate further *in vitro* (Fossard et al, 1977). Treating the branches with a fungicide/ insecticide before pruning or bagging, has also been tried in order to destroy eggs or fungal spores (Cresswell and Nitsch, 1975). But in both methods, the effect of the use of antibiotics and fungicides, on the growth of the explants is unknown. Spraying the buds with a mixture of antibiotics and harvesting newly sprouted shoots, has been found to be very effective, in reducing contamination rate (Enjalric et al, 1988; Hu and Wang, 1983 and Litz and Conover, 1978).

1.2.2 Micropropagation Technique for Trees

To date, shoot tips and lateral buds have been the most commonly used explants. In micropropagation, the use of larger explants is desirable, as they are easier to dissect and have much higher survival and growth rates. Considering the patterns of morphogenesis, the following micropropagation pathways can be distinguished.

(a). Axillary Budding (Shoot Tip and Bud Culture)

Explant ⟶ Axillary buds ⟶ Multiple shoots ⟶ Roots ⟶ Plantlets

This pathway is simply an enhanced release of axillary buds. This is a useful means of producing plantlets from young or mature trees, with a lower risk of genetic instability than by other routes. The advantage of using axillary bud proliferation from shoot tips, or bud cultures is, that the incipient shoot has already been differentiated *in vivo*. Thus, only elongation and root differentiation are required to establish a complete plant.

The use of shoot apex culture for the rapid clonal propagation of trees, has been developed for many plant species (Barghchi and Alderson,1983; Bhojwani et al, 1984; Brown and Sommer, 1982; Barnes, 1985;). Although, the proliferation rate of this method may be slower than the other two methods (adventitious budding and somatic embryogenesis), this method has rapidly gained popularity in recent years , since its genetic stability and easy adaptability to most plant species (Hu and Wang,1983).

In axillary shoot proliferation, exogenous cytokinins play an important role in overcoming the apical dominance of shoots, and to enhance the breaking of axillary buds. The greater success of this technique in herbaceous species is, partly due to the weaker apical dominance and partly due to the stronger root regenerating capacity of them.

Adventitious root formation can be induced quite readily in many herbaceous plants, but, it can be very recalcitrant in most woody species, specially from the mature trees (Hu and Wang, 1983).

(b).I. Adventitious Budding (Organogenesis)

Explant ⟶ Callus ⟶ Shoots and roots ⟶ Plantlets

In this pathway callus initiates meristems, which give rise to shoots and roots. Occasionally, a limited number of shoots also develop from pre-existing buds in the explant. Since this pathway involves the formation of callus, the frequency of genetic changes is increased, especially, in the form of polyploidization and aneuploidization resulting from mitotic abnormalities (Hu and Wang, 1983).

The range of genatic variation (somaclonal variation) of the plantlets formed, appears to depend on the growth behaviour of the explant, in response to the medium, culture conditions, and the duration of the culture period spent in the callus stage. Woody species are comparatively slow growing and less responsive to medium composition, including growth substances and, therefore, when only shoots are formed, they may have to be excised or separated and then induced to root under appropriate conditions. This method of propagation, has often been used in coniferous species, particularly for the manipulation of seeds or seedlings (Boulay,1987).

The main sources of explants are, cotyledons, hypocotyls, excised embryos, shoot tips, nodal buds and portions of internodes. Using adult materials, leaf primodia of dormant buds or needles, are often taken as explants. The initiation of meristemoids or buds has usually been possible, but the elongation of such buds has often been difficult. This technique has a great potential for multiplication of trees. But the problem of obtaining true-to-type genetic copies of known and desired genotypes, which is the main purpose of cloning, remains unsolved.

(b).II. Somatic Embryogenesis

Explant ⟶ Callus ⟶ Cells ⟶ Embryoids ⟶ Plantlets

This pathway is very easy to induce in certain species, while being difficult or impossible in others. Somatic embryogenesis was first recognized, in tissue cultures derived from the tap- root of carrot in the late 1950's (Steward,1958; Halperin and Wetherell 1964). Somatic embryogenesis has been observed in the *in vitro* cultures of over one hundred plant species

(Amirato,1983), but reproducible results have been obtained in only a few tree species including mango and citrus (Litz, 1985; Luts et al 1985).

This technique can be very useful in propagating trees, mainly because, if it is possible to obtain somatic embryos from a mature tree, it can be assumed that true rejuvenation has been achieved.

Somatic embryogenesis involves the induction of bipolar structures bearing both shoot and root meristems, and has an obvious advantage over the axillary budding and organogenesis for use in the mass cloning of agricultural crops. Clonal multiplication by this method involves the induction of embryos, either on the surface of the explant, on callus or, in a cell suspension. In terms of their utility, the formation of embryoids on callus or explant has not much advantage over adventitious shoots, since isolation of individual embryoids still has to be done manually. In suspension cultures, embryoids form free, and separate from other cells or tissues; such embryoids can be used as seeds to produce populations of single propagules, which should be highly amenable to large scale handling.

1.3 HEVEA BRASILIENSIS IN VITRO

Use of tissue culture techniques for *Hevea*, as an alternative method of clonal propagation, is of a considerable interest,since, it is known that the full capacity of the species is, far from being exploited.The major shortcoming of conventional propagation by bud grafting is, the inevitable rootstock / scion interaction; which exhibit a variation of growth and yield even within the same clone. It has been reported, that the mother trees produced by vegetative selection yielded 15-20 kg of dry rubber per tree per year,while grafted clones today give only about 4-6 kg per tree per year (Carron et al, 1989). It has been suggested that, the stock scion interaction could be due to the anatomical differences between the tissues of the stock and scion (Satchuthananthavale,1973a).

1.3.1 Seed and Embryo Culture

The low fruit set of hand pollinated flowers of *Hevea* (around 1-5%), is always the limiting factor in plant breeding programmes; hence, culture of embryos prior to anticipated early fruit drop,is of importance to the breeder.

Culture of seeds *in vitro* as an alternative to the present method of *Hevea* germplasm transfer, has been tried at Rubber Research institute of Malaysia (Annual rep, 1979). This technique was successful, and it has the advantage as an additional precaution against the introduction of undesirable pathogenes, like *Microcyclus ulei* into other countries. *Hevea* seeds are shortlived , and so, germplasm can potentially be stored through cultured seedlings or embryos (Paranjothi, 1987).

It has been found (Toruan and Suryatmana, 1977), that embryos without cotyledons could be grown into seedlings on Murashige and Skoog basal medium, and could be successfully transferred to soil. On the other hand Muzik had found (Muzik, 1956), that decotyledonized embryos would not grow into normal plantlets, while the development of the embryos with 2/3 of the cotyledons attached, was similar to that of the embryos with full cotyledons. At least one third of a cotyledon was found to be essential for the embryo to develop into a healthy plant.But the successful culture of embryos without cotyledons, has been reported by Paranjothi et al, 1975. This has been possible only on liquid media and no growth has been seen on solid media. More recently (Carron, 1981, Carron et al,1989), the germination of immature embryos isolated 3 - 3.5 months after pollination has been accomplished, with 60% success rate. He obtained 90-95% success with four month old embryos, that is one month before the fully mature stage. Immature fruits have first been sterilized by soaking in a solution of NaOCl (20% Cl) for 20 minutes and then by dipping in alcohol followed by flaming. Dissected embryos were grown in 1/4 MS medium with 2% sucrose. No difference has been observed either on solid medium or, in liquid medium (with support of a filter paper or vermiculite). Addition of growth substances (IAA,KIN),coconut milk or higher levels of sucrose (0.8-1.2%) had no effect. Embryos younger

than 2.5 months did not produce plants.

It has been reported (Normah et al, 1986),that the successful cryopreservation of embryonic axes in liquid nitrogen, and formation of seedlings with normal shoots and roots when cultured *in vitro*.

1.3.2 Axillary Budding

(a). Shoot Tip Culture

Juvenile origin. Root formation on 2-3 cm long shoot tips obtained from 2-3 week old aseptically grown seedlings, has been reported within four weeks of transfer into liquid media, consisting of casein hydrolysate and 3% sucrose (Paranjothi and Ghandimathi,1975b). But no growth has been reported on solid media. Plantlets have also been obtained from 1 mm long apices of seedlings (Carron et al, 1989).

Mature Origin. Failure of plantlet formation from shoot apices of clonal plants has been reported (Paranjothi and Ghandimathi, 1975b). Slight expansion of leaf primodia has been observed when the medium contained 1 ppm BAP and 1 ppm GA3.

Development of about three shoots per explant has been reported, when terminal buds from one year old budded stumps were cultured on solid MS medium supplemented with 0.5 ppm kinetin, 2 ppm BAP, 200 ppm casein hydrolisate, 0.1 ppm calcium pantothenate and 0.1 ppm biotin. Roots have been induced on 60% of explants on White's medium containing IAA, IBA, IPA, and NAA at 10 ppm each, for 72 hours, followed by transferring onto a solid medium containing 0.25% charcoal, for 10-15 days and then, into a liquid medium with no charcoal,for 10-25 days, before transferring to pots (Mascarenhas et al,1982).*Hevea* has been one of four species reported in this paper and no further report of progress has appeared so far.

(b). Node Culture

Juvenile Origin. Stem node culture has been demonstrated using young *Hevea* stem pieces of

green house grown plants (Carron and Enjalric 1982). Stem pieces of 3-4 cm long, containing one or more axillary buds, immersed in a mixture of hormone solution containing BAP and IBA at 10ppm and 5ppm respectively, for two hours, followed by culturing them on agar medium containing 5% charcoal and 6% sucrose, has resulted axillary shoot growth. Rooting has been possible, by dipping the bases of the isolated shoots in a solution of IBA and NAA, at 5ppm each for five days followed by culturing on a hormone free medium.It has also been reported, that 2-6% sucrose has improved the frequency of bud break and the quality of leafy shoots (Enjalric and Carron,1982). Activated charcoal at 5% encouraged the elongation of axillary buds with both 2% and 6% sucrose in the medium; Growth substances (BAP 2 µM, IBA 1.25 µM and GA3 1.44 µM)showed a beneficial effect on elongation of axillary buds, when the sucrose level is 2%, and a little or no effect at 6%.Rooting has been observed by dipping the bases of the shootlets in a solution of IBA (25 µM) and NAA (27 µM). The basal medium used was, the MB (Appendix A) solidified with 0.7% difco agar. Production of 4-6 plantlets from one explant has been reported by Carron et al,(1984),with successful acclimatization of plantlets. Rooting has been possible even with a commercial rooting powder, containing NAA. Transplanting of rooted plantlets to the field at success rate of 15-50%, has also been reported by the same authours (Carron et al, 1985). Monthly multiplication coefficient of 2-3 shoots has been reported using *in vitro* grown shoots (Carron et al, 1989).

Mature Origin. Difficulty in disinfection and bud release have been experienced with mature origin explants. Use of nodal cuttings from very young budded plants (Green budded) showed some bud opening, although they were not as strong as young material (Carron et al, 1984). Incorporating antibiotics into culture media (kanamycine 20 ppm; chloramphenicol 60 ppm; chlorotetra cycline 10 ppm)has been found ineffective in reducing contaminations (Carron et al, 1985). However plantlets have been obtained from the clones, PR 107, GT 1, PR 261, PB 86, PB 235, IRCA 438, IRCA 440 and IRCA 442. Spraying the mother plants with a mixture of antibiotics and fungicides,containing gentamycine 20 ppm, kanamycine 20 ppm,

chlorotetracycline 30 ppm, chloramphenicol 60 ppm, rifampicine 75 ppm, and benomyl 750 ppm, about 15 days prior to take explants, has been reported as a more effective method to reduce contaminations in culture (Enjalric et al, 1988).

1.3.3 Haploidy and Somatic Embryogenesis

(a). Haploidy

I. Anther Culture

The first successful anther derived callus culture, which could be serially subcultured was reported in early 1970's (Satchuthananthavale and Irugalbandara, 1972). The first production of haploid pollen plants in *Hevea* was reported in 1977 (Chen et al, 1979). Numerous papers have since been published, on anther culture work of rubber at various stages of success.

Male flower buds of clones RRIC 52 and KH 440 at various stages of development have been cultured (Satchuthananthavale, and Irugalbandara, 1972) on solid Nitsch and Nitsch (1969) medium supplemented with IAA, 2,4-D, GA3, Kinetin and coconut milk with 2% sucrose. It was found that the developmental stage of anthers was critical for callus formation. Good callus growth was maintained for about six months on the same medium by subculturing. It was proved by histological studies that cell proliferation occurred from the anther wall and connective tissue, but, not from the pollen grains (Satchuthananthavale and Irugalbandara,1972; Satchuthananthavale 1973a; 1973b).

Embryoid formation from anther derived callus on MS solid medium in the presence of 10% sucrose and 10% coconut milk has been reported (Ghandimathi and Paranjothi, 1975; Paranjothi and Ghandimathi, 1975a; 1975b). No shoot or root development of embryoids has been observed.

Toruan also reported callus growth from the somatic tissues of anthers on Nitsch and Nitsch medium consisting of 2,4-D, IAA and cytokinin at 0.1, 1.0 and 1.0 respectively with 15% coconut milk (Toruan and Suryatmana,1977).

However, Chinese workers produced pollen plants for the first time by anther culture in 1977 (Chen et al,1977). According to them, they all have had main roots, cyclic lateral roots, stems, cotyledons, first pair of leaves and terminal buds (Chen et al, 1979).

Generally induction of pollen plants is carried out in steps. The success of the procedure of anther culture partly depends on the incorporation of coconut water into the medium and partly on the use of sucrose at a level of 7-8%. Both the nitrogen and sucrose, and the concentrations are evidently important for the dedifferentiation of microspores (Chen, 1984). For callus induction 2,4-D and kinetin are sufficient. The time of transferring callus into regeneration medium is very important. NAA, GA3 and kinetin are needed in this medium. A high concentration of total nitrogen is required for the induction of pollen embryos, while a decrease of NO3 concentration of the medium, favours the procedure. Visible embryos emerged after about one month on this medium. Embryoid development was complete after about 2-3 months, when they were transferred to fresh medium for plantlet formation (Chen, 1984).

Determination of chromosome numbers in embryoids, plantlets and established plants, has been carried out and successive increase in the chromosome number in the developing embryoids, plantlets and transplanted plants has been observed. In embryoids, 9, 18, 27 and more than 27 chromosomes were observed. In root tips tested, the observed chromosome numbers were 9, 18, 20, 24, 27, 30, 36 and 45 (Chen et al, 1979; 1981; 1982; Chen, 1983; Cen et al, 1981).

II. Ovule Culture

Development of callus from cultured ovules of *Hevea* (clone Haiken 1), and subsequent formation of embryoids has been reported (Guo et al, 1982).Differentiation of both shoots and roots has been observed but, the success rate was only 1.1%, which is very low.

(b). Somatic Embryogenesis from Nucellus Tissue

It has been reported, that the callus generating potential of the maternal tissue in the seed, is

superior to that of any other part of *Hevea*. Induction of embryoids, from the callus derived from the fragments bearing inner integument and nucellus, has been reported (Michaux-Ferriere and Carron,1988 ; Carron et al,1989). For the callus formation, 2,4-D (0.3 ppm) in combination with IAA (1.0 ppm), has been found to be the best. BAP at 1-5ppm has promoted the callus proliferation. Increase of sucrose from 3% to 8%, has also increased the proliferation rate.Embryods visible to naked eye have been observed after 5-6 months on the differentiation medium supplemented with NOA and BAP each at 0.5 ppm and sucrose at 2%. Development of embryoids into plantlets has been achieved in a medium consisting of 5% activated charcoal and IBA at 12ppm, with a success rate of 1%. It has also been reported, that the embryoid formation on callus is cultivar dependent, and so far plantlets have been obtained only from the clone RRIM 623 (Carron et al, 1989).

1.3.4 Callus Culture

Although callus cultures of *Hevea* can usually be initiated without much difficulty from a variety of explants, callus cultures are not always amenable to subculture. However, the induction of callus from *Hevea* explants has first been documented in 1953 (Bouchou, 1953); this was the first reported tissue culture work in *Hevea*. Juvenile *Hevea* stems about 2-3 cm long, were grown on solid Knop's medium, supplemented with cysteine, pantothenic acid, biotin, inositol, glucose, NAA and IAA. Callus developed at the basal end of the explant, and latex has been observed in the callus. No embryogenesis or organogenesis has been reported.

It has been reported the induction of callus from *Hevea* plumule tissue using 3-4 days old seedlings (Chua, 1966). It was not possible to initiate callus on either, Gautheret's (1942, 1950) or White's (1943) media. Good callus growth has been obtained using a medium containing higher levels of media components. By increasing the sucrose concentration from 2% to 10% callus formation was enhanced. Good callus growth was observed when the pH of the medium was between 5.4-6.8. The type of callus produced,varied depending on the pH of the medium.

Root formation but no shoots, were observed in these calli, after about 5-6 months without subculturing. This was the first report on organogenesis of *Hevea* callus. Formation of callus from roots, cambial explants, cotyledon pieces, unfertilized ovules and stem segments has been reported on media, containing an auxin and a cytokinin (Paranjothi and Ghandimathi, 1975b). Latex has been observed in callus originated from epicotyls. Callus formed from both epicotyls and hypocotyls have formed roots.

1.3.5 Cell Suspension Culture

It has been reported that, the establishment of cell suspension cultures of *Hevea* using young stems as explants (Wilson and Street,1975 ; Wilson et al, 1976). Newly initiated cultures on solid medium consisting of 2,4-D 2ppm and kinetin 0.5ppm, produced roots. Serial subculture of the callus on solid media has not been possible. The callus was heterogeneous with brown necrotic tissue and soft white tissue. Subculturing this callus into liquid medium of the same composition, and then returning to solid medium, have resulted the production of homogeneous friable and rapidly growing callus. When the suspension were maintained for several months without subculture, the larger cell aggregates gave 'embryo like' structures. But the attempts to promote further development of these embryo like structures into plantlets has not been successful.

1.3.6 Protoplast Culture

Successful isolation of protoplasts from cell suspension cultures and, from pith tissue of *Hevea* has been reported (Othman and Paranjothi,1980). Pith-derived cells from suspension cultures, or pith tissue from young green shoots, were incubated in an enzyme solution containing 2% cellulase, 1% hemicellulase, 1% pectinase, 6.5% mannitol and 0.1% $CaCl_2.2H_2O$ at pH 5.5 for six hours at 34 -36 C for the release of protoplasts. Cell wall regeneration of isolated protoplasts, has been observed within 24 hours in culture, but the cell division has not been

observed. Protoplast from anther-wall derived callus are potentially useful, since, the callus has been shown to exhibit totipotency. It has also been found, that cell cultures derived from seedling-pith were the most suitable for *Hevea* protoplast cultures.

Fusion of protoplast of *Hevea brasiliensis* and *Hevea pauciflora* has also been reported (Cailoux and Lleras, 1979). However, fine suspension cultures were necessary for isolation of viable protoplasts. These were obtained by repeated serial subculture of the anther wall-derived callus in liquid media. (Paranjothi, 1987).

1.4. NATURAL RUBBER IN HEVEA BRASILIENSIS AND OTHER SPECIES

1.4.1 Occurence and Chemical Structure

Natural rubber,a polymer of isoprene (Fig.3) is known to be formed in over 1800 species of plants, distributed among 300 genera (Backhaus,1985).But commercial rubber is produced only from *Hevea brasiliensis* which yields about 1500 lb of rubber per acre per year.

Rubber is in general, produced in the plant in the form of microscopic particles (0.10-15 μm,diameter) in the cytoplasm of specialized cells : the latex vessels.Since latex occurs in the cytoplasm, it contains a mixture of low molecular weight metabolites, nucleic acids,proteins and other materials in addition to rubber.The rubber concentration in latex is very low in most rubber producing plants, except in *Hevea brasiliensis*; the concentration of rubber in *Hevea* latex can reach extraordinarily high values of 25%-40%,of which, 98% is cis-1,4-polyisoprene (Goodwin and Mercer,1983).

Rubber differs from the great majority of other terpenoids in two respects:

1.The molecular weight of rubber is high with a wide distribution, ranging from less than 10^5 to about $4x10^6$ Dolton.

2.The geometrical configuration about the double bond in the polyisoprene chain is, all *cis*, as judged by X-ray diffraction and nuclear magnetic resonance measurments (Archer and Audley,1967).

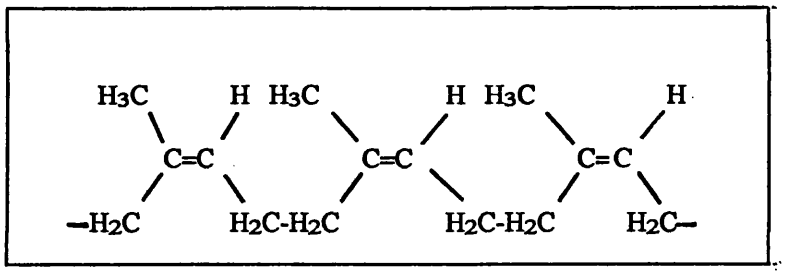


Fig.3. Part of a cis-1,4-polyisoprene chain.

In general the latex vessels which are located in the bark, form an interconnected anastomosing continuous system. In *Hevea brasiliensis*, latex is produced and stored in the inner cortex in laticifers, where it forms a major part of the cytoplasm.These specialized vessels are formed from chains of contiguous cells in the phloem,where they are arranged as sheeths concentric with the bark (Dickenson,1969). Between the vessels in each ring there are anastomers, which allow withdrawal of latex from a large area of bark by means of a single tapping cut. No such mechanism is operable in guayule, because, there are no laticifers in guayule (Bonner and Galston,1947). Among the many other plants, which produce polyisoprene latices, several have been considered as commercially valuable sources. One such is, guayule (*Parthenium argentatum*) which, like *Hevea*, produces cis-polyisoprene; but this has a lower molecular weight than that of *Hevea*, and its latex has the disadvantage of a high resin content (15%-20%). But, the lack of success of commercial guayule rubber production is mainly due to the lack of laticifers in guayule(Archer and Audley,1987).In order to extract the rubber from the tissue,the whole bush has to be destroyed.In guayule the rubber is produced in the cytoplasm of the parenchyma cells,but as the cells mature, the rubber particles appear to migrate into the vacuole, where they are stored (Backhaus and Walsh,1983 ; Goss,et al,1984). It has been found that, the bark and the root tissue had the highest potential for rubber production,while young stems had a lower potential (Macrae,et al,1986). Other species which have been of interest either

commercially or scientifically include *Castilloa* spp.,the vine *Cryptostegia grandiflora* and the Russian dandelion *Taraxacum kok-saghyz*. *Castilloa* can be tapped only a few times a year, because of the slow rate at which the rubber is regenerated. In *Cryptostegia*,latex can be collected more frequently from stems, but, each plant produces only a small amount of latex (Bonner and Galston,1947).

1.4.2 Biosynthesis of Rubber and its Regulation

The latex contains rubber as well as all the enzymes required for its biosynthesis and mitochondria for the generation of the ATP needed.After the cessation of latex flow from a tapped tree of *Hevea*, rubber is again formed. *Hevea* trees which are tapped every second day, can continue to synthesize rubber for almost the whole of their lifetime (Bonner,1967). It is clear therefore, that the rubber synthesizing system must be regenerated during the period between tappings.

The synthesis of enzyme molecules requires the presence of ribosomes in the latex; their presence has been demonstrated by McMullen(1962).All the amino acids required are also present in the latex as is ATP synthesised by the mitochondria also in latex (Andrew and Dickenson,1960). Evidence have been found for the presence of the isomerase enzyme on the surface of the rubber particles present in *Hevea* latex (Lynen,1969).

The renewal of latex requires the activity of nuclei. These are not removed from the latex vessels during latex flow and remain appressed to the vessel walls (Archer and Audly,1967; Benedict,1981; Bonner,1967), although the presence of some nuclei in *Hevea* latex has been reported by others (Archer,1980; Bonner and Galston,1947). Most of the genes in the genetic material of these nuclei are repressed; but the genes concerned with making of m-RNA for the enzymes of rubber synthesis are belived to be turned on all the time. The enzymes concerned with the biosynthesis of rubber must cease to act, once the rubber concentration has reached the required and predetermined level. The whole rubber making process appears to be regulated by

the concentration of rubber in the latex vessels (Bonner,1967).When the concentration is low the production of enzymes for rubber synthesis and rubber synthesis itself proceed at full speed. As the rubber concentration in the vessels approaches the maximum level, all processes, concerned with rubber making slow down and eventually stop (Bonner,1967). Therefore rubber synthesis in latex vessels depends on gene regulation of enzyme activity.

The effect of various chemicals on the production of rubber in *Hevea* and guayule has also been studied. In *Hevea*, ethylene stimulates the yield, and it has been suggested that the hormone may affect the genes responsible for the latex clotting mechanism, which leads to an increase of rubber production. It has been reported that, low night temperatures stimulate rubber production in guayule (Goss,et al,1984) and it has also been suggested that low temperatures stimulate transcription of the genes coding for the enzymes involved in rubber biosynthesis (Bonner,1967). Treatment with 2-(3,4-Dichlorophenoxy) - triethylamine has been reported to increase the rubber yield of guayule notably (Benedict,et al, 1983).

Biosynthesis of Rubber

The biogenetic isoprene rule (Ruzicka,1953), according to which, all the terpenoids have a common precursor,has been tested and confirmed by many others (Bonner and Galston,1947; Chaykin,et al, 1958; Lynen,et al, 1958). The wider field of isoprenoid biosynthesis which includes terpenes, sterols, bile acids, carotenoids and the ubiquinones, has been covered exhaustively, (Popjak and Cornforth,1960; Clayton, 1965), the subject as applied to rubber in particular, has also been disscused by several others (Archer,1950 ;Arreguin and Bonner,1950; Bonner,1960; Arreguin et al,1951 and Fournier and Cuong,1961).

The biosynthesis of rubber can be devided into three stages;

1. The generation of acetyl-CoA from acetate.
2. The conversion of acetyl-CoA to IPP via mevalonic acid.
3. The polymerization of IPP into rubber.

Studies on rubber biosynthesis has been carried out mainly with guayule. However, evidence has been found that, acetate forms the basic precursor for rubber biosynthesis in all rubber producing plants (Bonner,1960; Bonner,1967). When rubber producing plants are supplied with large amounts of uniformly labelled ^{14}C acetate, the acetate is incorporated into rubber and the carbon of the rubber formed, posseses a specific radioactivity equal to that of the acetate supplied (Arreguin et al 1951). This proves, that the carbon in rubber is essentially acetate. Biosynthesis of the isoprenoid monomer from the 2-carbon acetate molecule, has been explained as condensation of three acetate molecules with the elimination of one carbon atom (Bonner,1967). This involves the acyl transferring-CoA, which functions as a carrier in the process.

Another important step in understanding the biosynthetic pathway of rubber has been, the discovery of the fact that mevalonic acid is a precursor. It proved to be metabolized, to yield any and all isoprenoids including rubber (Park and Bonner,1958; Stanley,1958 and Purcell et al,1959). The transformation of mevalonic acid has been studied using *Hevea* latex (Williamson and Keckwick,1963.,Archer et al,1963).The isolation of mevalonic kinase from *hevea* latex has been reported. This enzyme catalyses the mevalonic acid in the latex of *Hevea* (Williamson and Kekwick,1963). It has also been reported that the incorporation of mevalonate into rubber was retarded in the presence of non-rubber particles (lutoid particles) in *Hevea* latex (Archer et al, 1963).

In the metabolic pathway from mevalonic acid to rubber isopentenyl pyrophosphate (IPP), has been identified by Archer et al (1963). IPP is then polymerized by an enzyme or enzymes to form rubber. In this function IPP is believed to be utilized at least ten times more rapidly than mevalonic acid (Archer et al, 1963).Isopentenyl pyrophosphate is also utilized by *Hevea* latex with 100% effiency, that is, all IPP supplied to the latex, is transformed to rubber (Archer et al,1961).

A general pathway has been suggested (Fig.4.) for the biosynthesis of isoprenoid compounds

(Barker,1971). In figure 4, only the final steps from IPP are specific to rubber biosynthesis. In the process of biogenesis of rubber, each new C_5 unit added, must assume the *cis* configuration. The configuration of each C_5 unit as it is added to the growing terpene chain, is presumably enzymatically determined. The cessation of the growth of the chain is believed to be associated with the absence of the polymerization enzyme for further growth. (Bonner,1967).

1.4.3 Determination of Rubber in Plant Materials.

(a).Chemical Extraction

Several different extraction methods have been reported with the use of acetone,petroleum ether, ethyl alcohol, benzene, carbontetrachloride, sulfuric acid etc.as solvents (Garrot et al,1981.,Macrae et al, 1986). In the method described by Hayman et al (1982),the samples are dried and ground and, interfering resinous materials are removed by Soxhlet extraction with acetone. The rubber is then extracted with benzene or methylene chloride overnight and, the residue weighed after evaporation of the solvent (Hayman et al,1982). It has been suggested that, before the extraction of rubber in guayule with hexane, samples need to be extracted with acetone for 24 hours for the removal of resins (Benedict et al, 1983). Dissolved rubber has then been evaporated to dryness and dissoloved in $CDCl_3$ for NMR analysis. The use of liquid nitrogen for freezing the samples prior to extraction, has been proposed as a method to reduce time required in the extraction procedures. Rapid freezing of plant cells and subsequent thawing, fracture the cells to allow rapid exposure of the rubber to the solvent without extensive grinding. At the same time, the frozen tissue can be easily milled without predrying (Garrot et al, 1981).

(b).Gravimetric analysis.

A rapid gravimetric method that yields very reproducible results has been reported for use in the determination of rubber in guayule. This involves a two step solvent extraction procedure, in which the ground guayule shrub is further subjected to the shear and sonification, generated by a

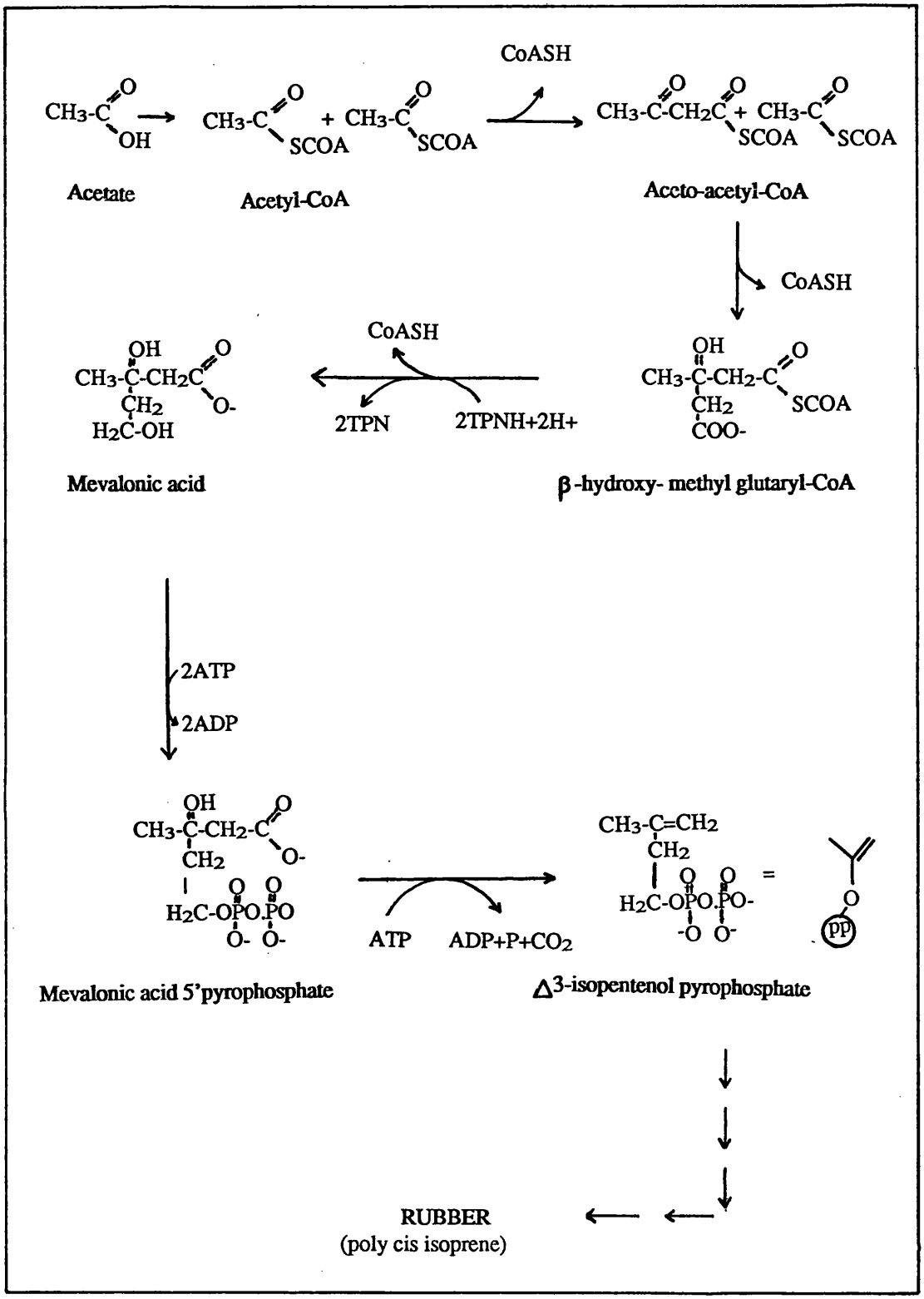


Fig 4. Biosynthetic pathway of natural rubber (Barker,1971).

high speed homogenizer grinder (Black et al, 1982). This allows both the initial resin fraction (acetone soluble) and the subsequent rubber fraction (cyclohexane soluble) to be removed efficiently. This grinding action in the mixture disrupts plant cell walls and renders the rubber immediately accessible to the solvent.

Using this technique, it has been shown that acetone removes 91% of the resins after the first extraction and the remaining 9%, in the second extraction. The cyclohexane has removed 96% of the rubber after first extraction, and the remaining 4% in the second extraction. No detectable resin or rubber, has been found in subsequent extractions.

Both resin and rubber fractions have been checked for cross-contamination by 13C-NMR. The resin fraction has been found to contain a small percentage of low molecular weight rubber. For the rubber fraction NMR analysis has yielded gamma values, agreeing with tipical isoprenoid carbon structures and, within the limits of detection no resin contaminants have been found.

It has also been reported that, high molecular weight rubber degrades in hot solvents, such as acetone during Soxhlet extraction. Rubber has been detected in the acetone fraction after 48 hour of Soxhlet extraction (Black et al, 1982).

(c).^{13} C Nuclear Magnetic Resonance Determination.

Determination of rubber in guayule using ^{13}C-NMR has been reported to be less time consuming, and more accurate than solvent extraction methods (Hayman et al, 1982). About 0.3g of dried ground material has been used in a ^{13}C Fourier Transform NMR spectrometer operating at 15 MHz (Hayman et al, 1982). Both *Hevea* rubber and rubber in guayule, has yielded spectra containing two peaks in the 12-13 ppm region and three peaks in the 20-35 ppm region (Hayman et al, 1982 ; Shoolery,1978). The NMR studies have shown that natural polyisoprenes are essentially 100% 1-4 structures and also that, the double bond in *Hevea* is at least 99% *cis* (Golub et al, 1962). It has also been reported that, although the ^{13}C NMR and Soxhlet extraction methods give resonably similar valus for the amount of rubber, figures obtained by NMR method

are consistently higher than those obtained by Soxlet extraction. This has been partially explained, by detecting 3-5% of rubber remaining in the plant residue of Soxhlet extraction method. Increasing of extraction time had not increased the amount of rubber.

1.4.4 Incorporation of Rubber Precursors into Rubber.

(a).Using Intact Plants

The possibility of increasing the amount of rubber accumulated by guayule seedlings, by incorporation of various substances, such as, acetate into the nutrient solution in which the plants were grown, has been demonstrated (Bonner and Arreguin, 1949).

Studies on the mechanisms of rubber formation in guayule using isotopic carbon, have been carried out by Arreguin et al (1951). It has been found that, when large amounts of ^{14}C acetate was given to the plants, about 10% of the radioactivity initially present in the nutrient solution was taken up to the plants (Arreguin et al,1951). Out of the total activity taken up as much as 44% has been recovered as rubber.

(b). Using Latex

Since *Hevea* is the only species from which latex can be obtained commercially, all experiments with latex,were carried out with this plant. Latex from *Hevea* must contain all the enzymes for rubber biosynthesis, because it has been demonstrated (Bandurski and Teas, 1957) that the ^{14}C supplied as acetate, pyruvate or methylcrotonate can all be incorporated into rubber in a latex preparation

^{14}-C labelled Incorporation of ^{14}C-mevalonic acid into rubber with a lower efficiency by incubation with *Hevea* latex has also been reported (Park and Bonner,1958). It has been shown that, only 10% of the mevalonic acid lactone was transformed into chloroform soluble polyisoprene of high molecular weight (Kekwick et al, 1959).

It has been found by Harris and Kekwick (1961) that, sodium [3^{14}C]-pyruvate is less

efficient a precursor than than sodium [2^{14}C]acetate. It has also been reported that, if the latex is obtained from the tree under a nitrogen atmosphere, and if the incubation is carried out in an atmosphere of nitrogen, the incorporation increased by 100%.

Conversion of mevalonate to IPP in the aqueous phase of *Hevea* latex through the intermediate of mevalonate-5-phosphate and mevalonate-5-pyrophosphate, has also been reported (Archer et al,1963). The incorporation has occurred at the surfaces of rubber particles in the latex.

Rapid incorporation of IPP into high molecular weight polyisoprene on incubation with *Hevea* latex has also been reported (Archer et al,1961 ; McMullen and McSweeney, 1966). Work has also been done on the influence of partical size of rubber on the incorporation rate (McMullen and McSweeney,1966). It has been reported that the smaller the partical size, the higher the specific rate of incorporation of IPP into rubber.

Archer et al (1961) have reported, that the addition of IPP to *Hevea* latex causes only chain lenthening of rubber molecules which are already present. Initiation of new chains has been reported to depend on the presence of dimethylallyl pyrophosphate in the system. It has been concluded that, the enzymes necessary for the utilization of IPP in rubber biosynthesis were present,both on the surface of the rubber particles and in the serum of the latex (McMullen and McSweeny,1966).

The incorporation of acetate and mevalonate into rubber using fresh latex from high, intermediate and low yielding clones of *Hevea*, has been studied (Woo and Edwin,1970).Both whole latex and the 'top fraction' of the centrifuged latex have been used. It has been reported, that individual variation, within any one clone, was too pronounced, and this makes the difference between clones insignificant or undetectable. Incorporation of acetate was found to be related to the annual yields of the clones, only when, the yields were distinctly different and when the whole latex was used, but not in incubation with 'top fraction'. These results confirms those of Archer et al(1963), who found that the 'bottom fraction' was essential for the conversion of

acetate into rubber. For the incorporation of mevalonate, not only, the bottom fraction is unnessary, but, it even inhibited the process (Woo and Edwin,1970).

Incorporation of acetate, mevalonate and pyruvate into latex at different concentrations of substrate, and the rate of the synthesis of rubber, has also been studied (Bealing,1975). It has been reported that, there is no considerable acetate pool in the latex and therefore, the percentage incorporation at high radioactive acetate give the actual rate of synthesis.

Addition of ATP (or phosphoenolpyruvate) to the incubation medium has been found to promote the incorporation of mevalonate into rubber, which is not surprising in view of the known ATP input for rubber biosynthesis process (see Fig.4.).

(c).Using Stem Pieces

Biosynthesis of rubber using isolated stem slices of guayule, has also been reported (Arreguin and Bonner,1950). The technique involves incubating of young stem pieces of guayule, under sterile conditions, on a nutrient medium in tubes, in order to obtain callus. The nutrient medium contained inorganic salts, sucrose, thiamine, pyridine, nicotinic acid, yiest extract and IAA. The resulting callus was then ground and analysed for rubber. In this experiment it has been shown that, the concentration of rubber found in the tissue does not change during the culture period of 3-7 weeks.

An extract of guayule leaves has been incorporated into the culture medium of stem pieces, and a marked increase in rubber formation in the presence of leaf extracts has been found. The leaf extract could contain substrate for rubber formation, or other factors which in some way stimulate the rubber forming system in the isolated stem tissues.

Among the variety of substances tested, acetate, acetone and methyl-crotonic acid, have been found to increase the rubber yield of stem calli.

Hevea stem slices have been used in an experiment to compare the efficiency of incorporation of two types of ^{14}C labelled acetate;methyl labelled and carboxyl labelled

(Rabinowitz and Teas ,1960). It has been reported that, the ratio of incorproration efficiency of methyl labelled acetate and caboxyl labelled acetate, varies with the length of the incubation period. For shorter periods like 4,6 or 8 hours the ratio was 1.5:1, which consistent with the concept of 3 methyl carbons and 2 carboxyl carbons forming the isoprenoid unit of rubber.

An efficient method for determination of rubber producing potential has been established, using young guayule stem pieces (Macrae et al, 1986).1-2 g of very thin slices, taken from the first 5 cm of young guayule plants, has been incubated with 10 ml of radioactive precursor in 1M phosphate buffer at 26°C for 16h in light. At the end of the incubation period the reaction has been terminated, using 80% boiling ethanol.

The ground sample has then been extracted with water,acetone and petrolium ether in succession, in a Soxhlet apparatus for 8 hours with each solvent. Radioactivity has been measured using a Beckman 3000 instrument.

Acetate, acetyl-CoA, mevalonate and isopentenyl pyrophosphate have been used as precursors, and it has been found that the incorporation of the first two substrates was significantly higher than that of the other two.

1.5 Aims of the Present Work

The objectives of the work reported here were as follows:

1. To work out a continuous micropropagation procedure for juvenile materials of *Hevea*. Even this apparently simple problem was outstanding at the start of this research, although plantlets have already been produced from juvenile materials such as nodes from seedling shoots (Carron et al, 1985).

2. To devise a suitable protocol for the micropropagation of elite mature trees of *Hevea*. A propagation method capable of producing true-to-type plants is a first priority of the whole rubber industry, because the known capacity of the species is far from being exploited today in the plantations.

3. To induce adventitious somatic embryos from young leaf lobes. This has already been worked out for cassava (Stamp and Henshaw, 1982) which is one of the Euphorbiaceae,i.e. the same family as *Hevea*.

4. To study the rubber producing potential of clonal plantlets of *Hevea in vitro* as determined by the rate of incorporation of rubber precursors, such as ^{14}C-acetate into rubber by stem slices as a means of preselecting superior genotypes.

Chapter. 2
Materials and Methods

2.1. Axillary Shoot Proliferation and Somatic embryogenesis.

2.1.1. Plant Material

Shoot tips and nodes from both juvenile and mature origin plants were used in the present studies

(a). Juvenile Plant Material

Seed Embryo Derived material

Mature seeds obtained from Rubber Research Institute of Sri Lanka, and Rubber Research Institute of Malaysia, were used to produce seedling plants *in vitro*, by embryo culture. Solid M&S medium at full strength, supplied with 6% sucrose, 0.5 ppm BAP, 0.5 ppm kinetin and 2 ppm GA_3 was used to culture embryos.They were grown in tubes, and the shoot tips and cotyledonary nodes were harvested from the resulting plants (Figure 5 a).

Root Stock Derived Material

Budded stumps grown in the glass house, give a reasonable amount of seedling shoots (Figures 5,b&c) which should be removed for the growth of the clonal bud. This type of shoot production could be successfully enhanced by spraying the stock plants with a solution of 2% thiourea, 1% KNO_3 and 100 ppm GA_3.

(b). Mature (Clonal) Material from Selected Clones

Budded stumps of clones PB 86, RRIC 100, RRIC 110, RRIC 117 and RRIC 121 obtained from the Rubber Research Institute of Sri Lanka, were grown in 9" by 20" polythene sleeves, in peat based compost mixture with medium nutrients (M2),supplied by Fisons,U.K. Plants were kept in the glass house at 25±2°C, 50-60% relative humidity. During the winter artificial light was provided to give a day-length of 12 hours. Terminal shoots were cut down after the growth of the 1^{st} or 2^{nd} leaf whorl, in order to enhance the development of new lateral shoots (Figure 5 d). The main stem was sprayed with thiourea mixture where necessary, to encourage lateral shoot growth further,and the plants were pruned regularly to obtain maximum number of new branches.

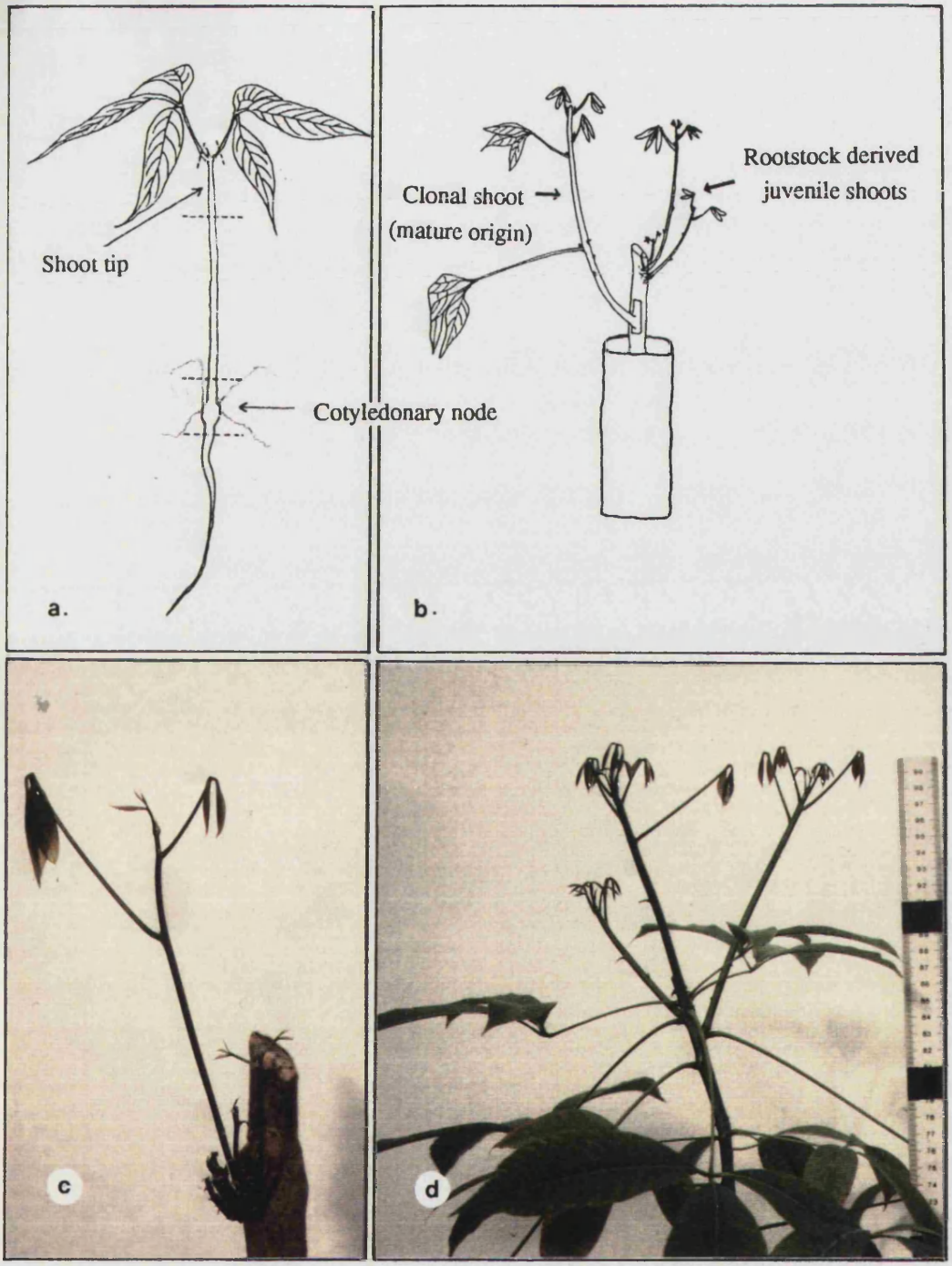


Fig 5 (a). Embryo cultured plant (the shoot tip and cotyledonary node), (b). Juvenile shoot growth on seedling rootstock and the clonal shoot,grown out from the grafted bud,(c).Juvenile

shoots as shown in (b), and (d).Clonal shoot growing into three shoots, when the main stem was polarded.

(c). Cotyledons and Young Leaves

Mature seeds supplied in batches from the Rubber Research Institute of Sri Lanka, were used to excise cotyledons.

Young leaves were harvested from *in vitro* grown cultures. They were grown on hormone free solid media for at least 3 weeks, before the leaf explants were harvested.

2.1.2. Media

(a). Chemicals-

Murashige and Skoog (1962) medium supplied by Flow Laboratories was used. BDH chemicals (Analytical Grade) were used in the preparation of stock solutions for woody plant medium (Lloyd and McCown,1980). Hormones used were from Sigma Chemicals Co Ltd. Agar used was, Lab M type MC2. Analytical grade sucrose, water soluble PVP (molecular weight 44,000) and activated charcoal were supplied by BDH.

(b). Stock Solutions-

For WPM medium 1 liter concentrated stock solutions of major elements including iron salts (excluding $Ca(NO_3)_24H_2O$), minor elements and vitamins were made up as shown in Appendix.2. $Ca(NO_3)_24H_2O$ was prepared as a separate stock solution, to prevent precipitate formation in the presence of the other majour elements. Stock solutions of hormones were prepared at a concentration of 100 ppm unless otherwise stated. These were first dissolved in a minimum amount of solvent (Appendix.3) and made up with distilled water to the required volume. The PVP stock solution was made up at a concentration of 1%. NaOH and HCl solutions at 1M and 0.1M were used to adjust the pH of the media.

Vitamin solutions were stored at $0^{\circ}C$ and all other stock solutions except NaOH and HCl,

were stored at 4°C. Hormones were prepared fresh every 4-5 weeks.

$HgCl_2$ solution was made at 0.2% for sterilization and stored at room temperature.

(c).Preparation of Media-

M&S basic medium was prepared by dissolving the powder in about 500 ml of distilled water. Woody Plant Medium was made by adding appropriate volumes from the stock solutions. Hormones were then pipetted in to the medium while stirring. Sucrose was used at 2% concentration and PVP was used at 100 ppm, unless otherwise stated. Required volume was obtained by adding distilled water and then the pH of the medium was adjusted to 5.7 using NaOH and HCl before adding agar at 0.6%.

When making solid media in tubes, they were boiled with agar and poured in to tubes, before autoclaving. For Petri dishes,jars and square wells, media were autoclaved in 500 ml glass bottles, or in flasks covered with aluminium foil, with the required amount of agar, and poured in the laminar flow cabinet.

When charcoal was used, pH was adjusted, after charcoal was incorporated into the medium. For solid media, the amount of agar had to be increased, according to the concentration of charcoal in the medium (Table 1.).

% of charcoal	% of agar
0.1	7
0.5	8
5.0	10

Table 1. The amount of agar required for different concentrations of charcoal.

Liquid media were prepared in tubes, and a paper wick made with a 5 cm Whatman No.1 filter parer, was used in the medium, to support the explant.

2.1.3. Culture Vessels

(a). Petri Dishes, Jars and Square Wells

Sterile Petri dishes of 15 cm, 9 cm and 3.5 cm diameter, supplied by sterillin Products, U.K.,were used with appropreate amounts of medium.

Sterile jars of 100 ml capacity with metal screw lids supplied by the same were used with 25-30 ml of media.

Square wells,consist of 25 squares were used with 4 ml of medium in each well, for the experiments with young leaf lobes and cotyledons. 25 different combinations of hormones were used in one square well.

(b). Glass tubes

16 x 200 mm Pyrex tubes with polypropylene closures were used with 15 ml of media.Slants were made where necessary, to give a maximum surface for shoot tips and nodes.Tubes were sealed with parafilm to prevent evaporation of medium.

2.1.4. Sterilization

(a). Plant Materials

Seeds- The seed coat was removed using a pair of pliers, and they were then surface sterilized in batches of about 100, in flasks. 10% solution of commercial NaOCl (14-15% available chlorine) with a few drops of Tween 80, was used, for 20-25 minutes, followed by 5-6 washes with sterile distilled water.

Shoot tips and Nodes- All the leaves and petioles were removered from the stems, harvested from the stock plants grown in the glass house. Shoots were cut into 4-5cm pieces, and washed thoroughly until all the latex and rubber particles were washed off. Surface sterilization was done

in plastic sterile jars, or in sterilized glass jars(175 ml capacity). Explants were washed in 70% EtOH for 1 minute prior to sterilization in $HgCl_2$, and then they were soaked in 0.2% $HgCl_2$ for 10 minutes with 1-2 drops of Tween 80. Explants were shaken throughout, and washed several times with sterilized distilled water, after $HgCl_2$ treatment.

(b). Media, Water and Instruments

Media and Water- Media and water were sterilized in an autoclave, at 121°C under 15lb/inch^{-2} for 15 minutes, in glass bottles with plastic screw caps.

Instruments- Forceps and scalpel blades were sterilized by dipping in absolute alcohol followed by flaming. Sterile blades were used in dissecting seeds and stems. For leaf culture sterile needles were used.

2.1.5. Culture Procedure

Embryo Culture- Sterilized seeds were soaked in sterile water for 24 hours before excising embryos. Dissected embryos with 1/4-1 cotyledons attached, were cultured onto solid media in tubes.

Shoot tip and Node Culture- After sterilization of shoots, about 1cm piece from the base of each shoot tip explant,and two pieces from either sides of the nodes, were trimmed before placing them on media. Explants, 2-3cm long were placed horizontally on solid media in Petri dishes. Shoot tips and cotyledonary nodes, harvested from seed embryo derived, *in vitro* grown, seedlings were also cultured, but without sterilizing or trimming.Cultures were transferred to fresh media every 4 weeks unless otherwise stated.

Cotyledon and Young Leaf Lobe Culture. Mature seeds were dissected as for embryo culture and cotyledons were separated into two halves before placing them on the media. Both abaxial and adaxial sides were cultured. 2-5 mm long, unexpanded, young leaves were removed from *in vitro* grown cultures, under the microscope. They were placed on the surface of the solid medium made in Petri dishes or square wells.

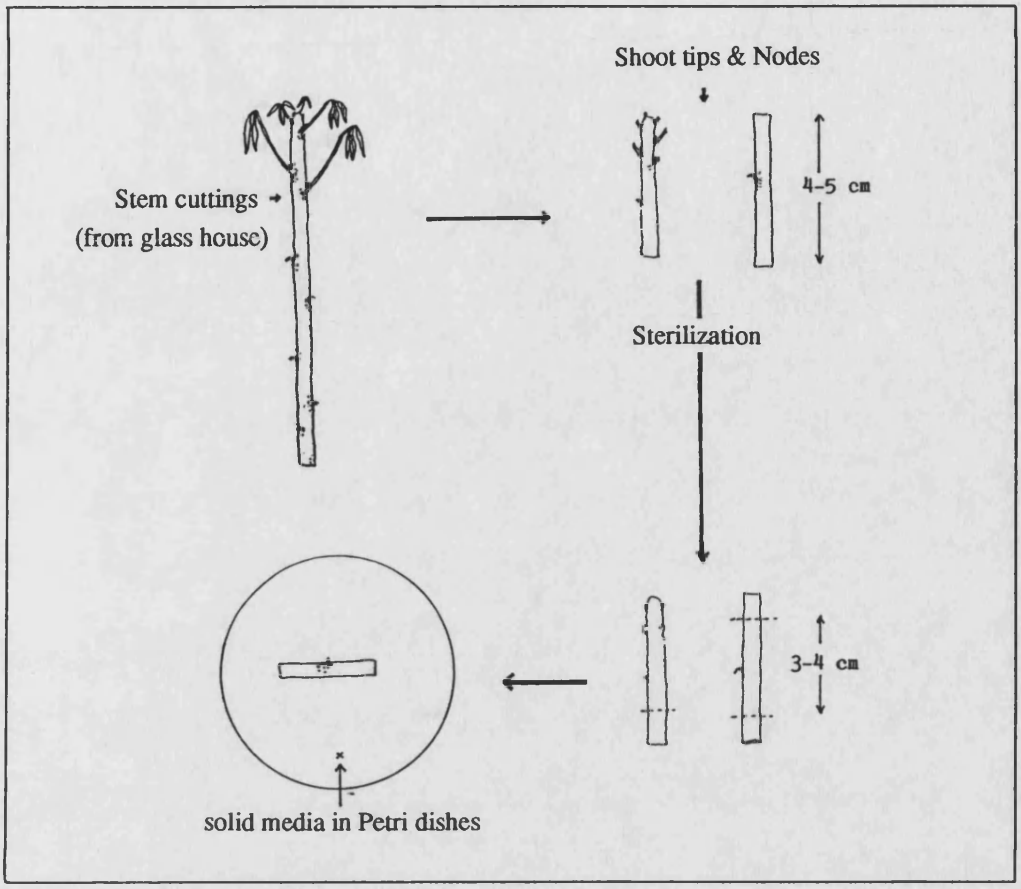


Fig 6. Culture procedure for shoot tips and nodes, of juvenile or mature origin, harvested *in vivo*.

2.1.6. Culture Conditions

All the cultures were incubated at 25±2°C, under a 16 hour photoperiod, at irradiance of about 100 $\mu E\ s^{-1}m^{-2}$ supplied by cool white fluorescent tubes, in growth chambers.

2.1.7. Statistics and the Calculation of Shoot Doubling Time

8-15 replicates were used for each treatment, and the standard error of the mean was calculated.

Shoot doubling time was calculated (Flegmann and Wainwrite,1981) as follows:

$N_t=N_0e^{kt}$ where N_t=Number of shoots at time t, and

N^0=Number of shoots when t=0.

k is the shoot proliferation rate constant. k was calculated from the plot of $\Sigma \ln N^t/N^0$ against t (time). The proliferation rate constant was converted to a shoot doubling time as follows: $t_d=\ln 2/k$

2.2. Incorporation of ^{14}C-Acetate and ^{14}C-Mevalonate into Rubber, in Stem Slices of *Hevea*.

2.2.1. Plant Material

Stem cuttings from plants, that have already been used for axillary shoot proliferation experiments, were used again for the experiments here. Both clonal and root stock derived materials were used.

2.2.2. Chemicals

(U-^{14}C) NaOAc (aqueous) and (2-^{14}C) mevalonate DBED salt (solid) supplied by Amersham,U.K., were used as rubber precursors. Mevalonate was dissolved in distilled water. Enzymes,cellulase and pectinase, were supplied by Yakult Honsha,Co.Ltd, Tokyo, Japan. All other chemicals including acetone and petroleum ether were analytical grade, supplied by BDH Chemicals, U.K. The scintillant 'Optiphase Safe',was supplied by FSA Laboratory Supplies, England.

2.2.3. Sample Preparation

Both young branches of actively growing shoots (top 5 cm of the shoots), and mature shoots (shoots in the resting state of their growth) were used for preparing samples. All the leaves and

petioles were removed, and the stems were cut into 0.5-1.0 mm thick slices in a Petri dish containing distilled water. Stem slices were mixed to reduce unwanted variation. Water was drained and the slices were dried on filter paper. Each sample consisted of approximately 2 g fresh weight, weighed to the nearest centigram. The whole procedure was carried out in the shortest possible time.

2.2.4. Incubation Medium

Each replicate contained activity of about 5.3 X 10^6 DPM. in 10 ml of solvent. Either distilled water or 5 µM $CaCl_2$ was used as incubation medium.

2.2.5. Incubation

2 g of stem slices were incubated with 10 ml of one of the two precursors, in 50 ml conical flasks, at 25°C in light in a shaker for 24 hours.At the end of the incubation period,the reaction was stopped by washing the dishes 3 times with boiling 80% ethanol or distilled water. When ethanol was used, samples were washed 3 times with cold water following the ethanol wash. Samples were then incubated with a solution of, 1% each of Cellulase and Macerozyme, for another 24 hours under the same temperature and light conditions, unless otherwise stated.

2.2.6. Extraction and Sampling

After treatment with enzymes samples were washed 3 times with cold water and transferred into Soxhlet thimbles. When mature shoots were used, stem slices needed maceration after enzyme treatment, before transfer into the thimbles. Thimbles, hand made from Whatman No.1 filter papers or proprietary cellulose extraction thimbles (10X50 mm, supplied by Whatman) were used. Plant material was then extracted in a Soxhlet apparatus for the appropriate lengths of time, with 50 ml of water first, then acetone and finally petroleum ether(boiling range 40-60°C).

At the end of each extraction time 1 ml of sample was measured out into a scintillation vial,

where 9 ml of scintillant ('optiphase safe') was added. The total volume of the solvent was determined either by measuring or weighing the flask (volume(v)=weight(w)/density(d); for acetone d=0.79 and for pet ether d=0.64) Sample and scintillant were mixed thoroughly and the vials were kept in the dark until radioactivity was measured in a liquid scintillation counter (LKB Wallac 1217).

2.2.7. Determination of ^{14}C Incorporation

The radioactivity recovered in each fraction was subsequently expressed as a percentage of the total uptake and also as a percentage of the total radioactivity recovered.

2.2.8. Treatment of Errors.

To compare clones, samples were taken from about 8-12 plants (depending on availability). As ponted out already stem slices were mixed thoroughly before weighing out 2 g of dishes for incubation with precursor.

Three or four replicates were used for each treatment and the standard errors of the means were calculated.

Errors were combined as follows:

The error **c** on a sum or difference, **C=A+B** or **C=A-B**, was calculated as,

$$c=\sqrt{a^2+b^2}$$

where **a** and **b** are the errors on the measured means **A** and **B**.

The error **c** on a product or quotient, **C=AXB** or **C=A/B** was calculated as

$$c=C\sqrt{(a/A)^2+(b/B)^2}$$

where again **a** and **b** are the errors on the measured means of **A** and **B**.

Chapter. 3
Micropropagation of Juvenile Materials of *Hevea.*

3.1. Results

The aim of the work presented in this chapter, was micropropagation of juvenile materials of *Hevea* by means of axillary shoot proliferation.

Both shoot tips and nodal cuttings were used as explants. Plant materials were derived from *in vitro* grown embryo cultured plants and glass house grown rootstock plants. Explant type, basic medium, sucrose level in the medium and the type of cytokinin were among the variables tested.

3.1.1. Axillary Shoot Production Potential of Shoot Tip Explants.

Experiments were carried out with shoot tips of both seed embryo and rootstock origin plants, in order to obtain axillary shoot proliferation. Shoot tips were chosen as explants, because they contained more axillary buds compared to a same length of shoot from the rest of the stem.

(a). Seed Embryo Derived Shoot Tips.

3-4 cm long shoot tips removed from 1-2 month old *in vitro* grown seedlings were used in this experiment. Half strength solid M&S medium supplied with 2% sucrose was used with four combinations of kinetin and BAP designated as S-0, S-1, S-2 and S-3. NAA was used at 0.2 ppm. Hormone compositions were as follows:

	Designation of medium			
	S-0	S-1	S-2	S-3
Growth hormone (ppm) kinetin	0	2	7.5	10
BAP	0	1	3.75	5
NAA	0	0.2	0.2	0.2

These four media were used in preliminary experiments, because S-2 medium proved to be the best, in earlier experiments on shoot tips of mature materials by the author. Media were prepared in 9 cm Petri dishes and cultures were transferred onto fresh media every 4 weeks.

Percentage axillary bud break, axillary shoot growth and leaf growth, were evaluated at 4 weeks intervals. Results for the first 12 weeks of culture are shown in Table 2.

Axillary bud break was observed within 4 weeks of culture in all four media. Most of the seed embryo derived shoot tips contained only two axillary buds, located just below the shoot apex. Apical leaf growth was observed in all cultures.

Even after 8 weeks, the lengths of the axillary buds remained less than 2 mm, irrespective of the medium composition. All the shoot tips grown on control medium, showed an apical elongation of about 5-10 mm and contained good leaf growth.

Leaf growth was scored as follows;

+ - very poor leaf growth
++ - poor leaf growth
+++ - good leaf growth
>+++- very good leaf growth

	no.of weeks	S-0 medium	S-1 medium	S-2 medium	S-3 medium
Percentage axillary bud break	4	5	33	75	60
	8	13	66	83	75
	12	13	66	85	75
Mean length of axillary buds (mm)	4	2±.5	2.5±.3	1.5±.1	1±.01
	8	2±.5	2.5±.3	1.7±.14	1.5±.02
	12	2±.5	3±.35	1.8±.11	1.5±.02
Leaf growth	4	++	++	++	+++
	8	++++++	+++++	+++	++
	12	++++++	+++++	++	+

Table 2. Percentage axillary bud break, mean length of axillary shoots and leaf growth on shoot tip explants derived from seed embryos. (n=9).

After 12 weeks, shoot tips on S-0 medium produced roots. Leaf growth maintained as good on both S-0 and S-1 media. Defoliation was observed in cultures on S-2 and S-3 media. Even

the shoot tips turned yellow on S-3 medium.

Therefore, no axillary shoot proliferation was observed with shoot tip explants of seed embryo origin. Plantlets could be obtained within 8 weeks of culture on control medium, but without any multiplication.

It was possible to maintain the cultures as shoot tips on both S-1 and S-2 media but those on S-3 medium could not be maintained beyond 16 weeks; they turned yellow and eventually died.

(b). Root Stock Derived Shoot Tips.

In this experiment shoot tips were harvested from the plants grown in the glass house. After sterilization, shoot tips of 4-5 cm were cultured as described for seed embryo derived shoot tips. Results for the first 12 weeks of culture are given in Table 3. Leaf growth was scored according to the scale given for Table 2.

	no.of weeks	S-0 medium	S-1 medium	S-2 medium	S-3 medium
Percentage axillary bud break	4	8	22	37	55
	8	50	54	42	60
	12	50	54	46	65
Mean length of axillary buds (mm)	4	1±.01	2±.5	1.1±.3	1±.2
	8	1.7±.1	2.9±.7	2±.1	2.5±.4
	12	1.8±.1	2.9±.7	3±.2	2.5±.4
Leaf growth	4	++	++	++	++
	8	++	+++++	+	+
	12	+++	+++++	+	0

Table 3. Percentage axillary bud break, mean length of axillary shoots and leaf growth on shoot tip explants derived from rootstocks (n=9).

After 4 weeks of culture, axillary bud break was observed on shoot tips in all 4 media at various rates. During the first four weeks, the percentage axillary bud break increased with the

increasing amount of cytokinins in the medium. This difference was unseen after about 8 weeks. The length of the axillary buds produced, remained less than 3 mm in all 4 media during the experimental period.

Good leaf growth was observed in S-0 and S-1 media, while very poor leaf growth was observed in S-2 and S-3 media.

As with shoot tips of seed embryos, shoot tips originated from rootstocks too, were defoliated and turned yellow on S-3 medium. About 75% of the shoot tips on S-0 medium and about 15% of those on S-1 medium produced roots.

Both seed embryo and rootstock derived shoot tips responded in the same way to the media tested in all aspects. No axillary shoot proliferation was obtained with shoot tips derived from rootstocks either, and the experiment was therefore terminated.

3.1.2. The Effect of Explant Type on Axillary Shoot Production.

Although shoot tip explants are generally considered to be better than nodal explants, no proliferation could be obtained with shoot tip explants of *Hevea* in the present work. This experiment was therefore designed to compare the axillary shoot producing potential of shoot tip explants with that of nodal explants.

Only rootstock derived explants were used in this experiment. M&S solid medium at half strength was used with 2% sucrose and S-1 hormones (kinetin 2 ppm, BAP 1 ppm and NAA 0.2 ppm). Media were prepared in 9 cm Petri dishes. There were 10 replicates from each type of explants, and cultures were transferred onto fresh media every 4 weeks.

Both shoot tips and nodes were 4-5 cm long, when cultured. After 4 weeks, nodes were cut into pieces, each containing only one axil. Axillary shoots produced were subdivided into propagules; each was either a node approximately 5-10 mm long with at least one axil, or a shoot tip. Axillary bud break and elongation after 12 weeks of culture on two types of explants are shown in Plate 2.

a. b.

Plate 2. Axillary shoot production on rootstock derived (a) nodes and (b) shoot tips. Results after 8 weeks.

Mean number of propagules produced per explant and the mean lengths were recorded at 4 weeks intervals. Results for the first 28 weeks of culture are shown in Figures 7 a&b (see also Appendix 4 a&b).

After 4 weeks of culture, 100% axillary bud break was observed with nodes. Within 12 weeks, some of them showed about 25 mm shoot elongation and good leaf growth (Plate 2 a). After 20 weeks of culture, axillary shoots grown on nodes were divided into propagules (nodes). After 4 weeks, new nodes showed axillary bud break at 100%. But, these secondary axillary shoots produced, were thin, slow growing and contained very small leaves compared to primary axillary shoots.

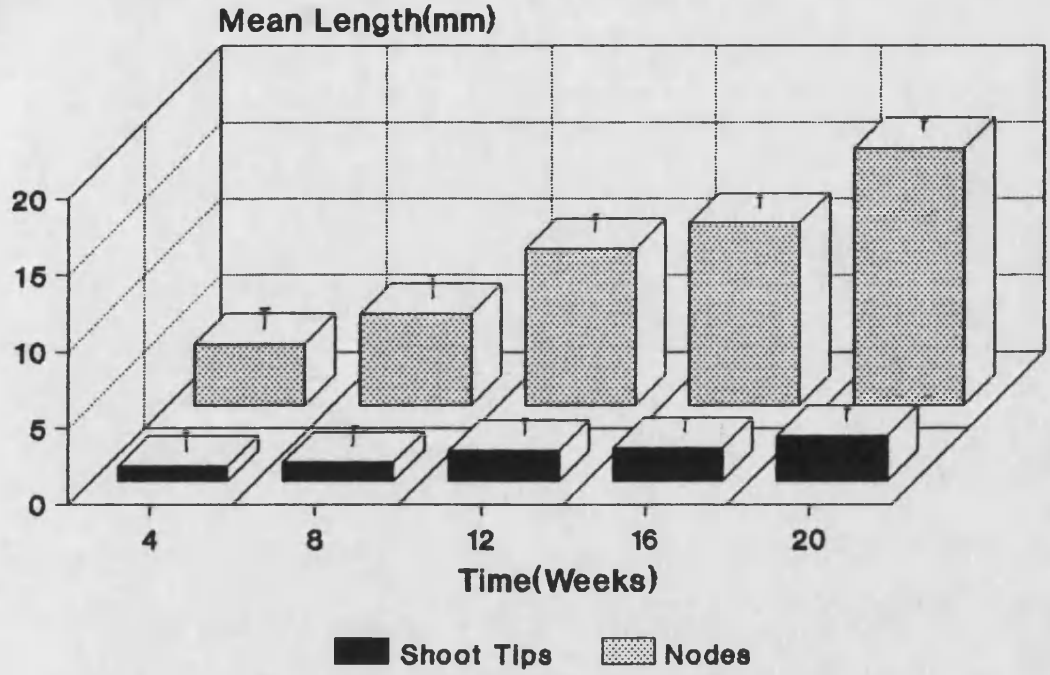


Fig 7 a . Mean lengths of axillary shoots produced by rootstock derived nodes and shoot tips (n=10). Cultures were recultured every 4 weeks.

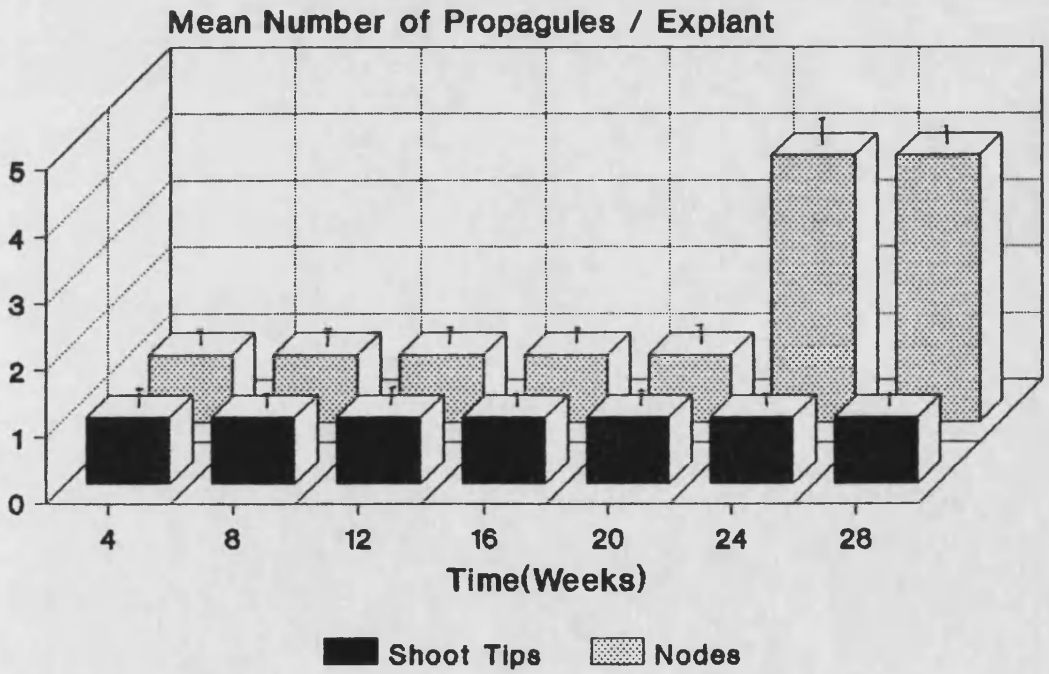


Fig 7 b . Mean number of propagules per explant, of rootstock derived nodes and shoot tips (n=10). Cultures were recultured every 4 weeks and the first subdivision was at 24 weeks.

Results obtained with shoot tip explants confirmed those of the previous experiments with shoot tips of both seed embryo and rootstock origin. After 4 weeks, only half of the originally visible axillary buds grew out from the shoot tips. Even after 20 weeks, axillary buds remained less than 3 mm in length. No leaf growth was observed either on apex or on axillary buds (Plate 2(b)).

Results of this experiment have showed that the axillary shoot production of nodal explants is much higher than that of shoot tip explants.

In order to see if there is any difference between the nodes derived from rootstocks and seed embryos, 10 nodes of seed embryo derived were cultured onto the same medium as before.

According to expectations, about 75% of the axillary buds were grown out within 4 weeks of culture. Cotyledonary nodes of seed embryo plants,carry two axillary buds on either sides of the node and, in most cases, both axillary buds produced axillary shoots with normal leaves. At the end of 4 weeks, the mean length of the axillary shoots produced was about 1 cm. After 12 weeks, axillary shoots were cut into nodes of about 1-1.5 cm. These nodes contained 4-6 axillary buds, and again, the growth of these secondary axillary shoots, was very slow compared to the growth of primary axillary shoots.

3.1.3. Optimizing the Medium for Axillary Shoot Proliferation of Nodal Explants.

(a). Combinations of kinetin and BAP.

This experiment was carried out only with rootstock derived nodal explants. The four media, S-0, S-1, S-2 and S-3, used in the experiment with shoot tips, were used, and compositions of which are given in page 49. Cultures were initiated in 9 cm Petri dishes as in the previous experiment, but they were transferred into 15 cm Petri dishes after the first passage.

The mean lengths of axillary shoots produced in each medium are shown in Figure 8 (see also Appendix 5.a).

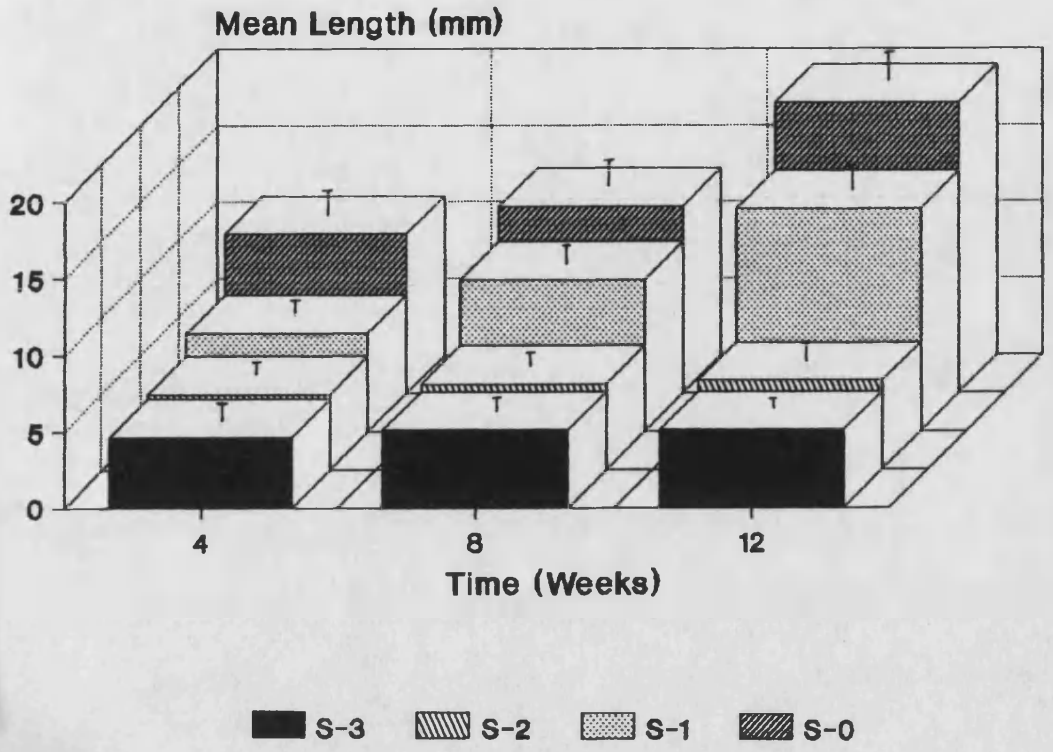


Fig 8. Mean lengths of axillary shoots of nodal explants on four combinations of kinetin and BAP (n=13). Cultures were recultured every 4 weeks and the first subculture was at 12 weeks.

Up to 12 weeks of culture, no significant difference was observed between media in the production of axillary shoots. Each node produced a single axillary shoot; but the lengths varied from 1-30 mm. About 60% of the axillary shoots produced on control medium, and about 40% produced on S-1 medium, were 15-30 mm long. But the maximum axillary shoot elongation observed on S-2 and S-3 media, was 20 mm, which was observed only in a few cultures. About 15% of the cultures showed 10-15 mm elongation of the axillary shoots.

As Figure 8 shows, the mean length of axillary shoots produced on control medium was the highest until they were cut into nodes at the end of 12 weeks. However, the leaf growth of axillary shoots on control medium was poor, although the stems were dark green and healthy.

Internodal expansion of the axillary shoots on S-1 medium, was also satisfactory, and a good leaf growth with normal triforliate morphology was observed throughout the experiment. The internodal expansion of the axillary shoots produced on S-2 and S-3 media, was significantly smaller than that observed on the control and S-1 media. The leaves produced on S-2 medium were 'needle like'and pale green. This effect was more pronounced on S-3 medium, where not only leaves but even the stems of the axillary shoots were very pale green. Root formation was observed on both primary and secondary nodes grown on control medium occasionally.

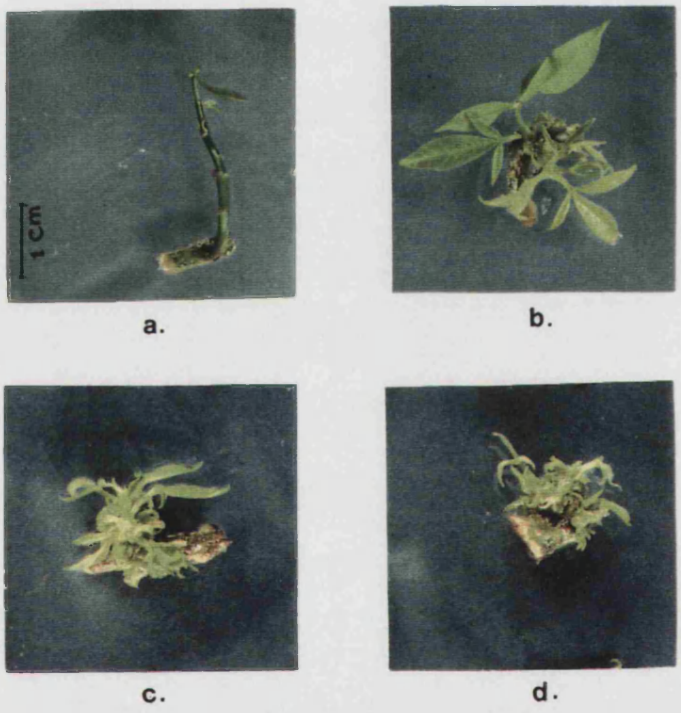


Plate 3. Axillary shoot growth on four combinations of kinetin and BAP, (a) S-0 medium, (b) S-1 medium, (c) S-2 medium and (d) S-3 medium.

Shoot proliferation of nodal explants on the four media, are shown in Figures 9 a, b, c, & d (see also Appendix 5 b).

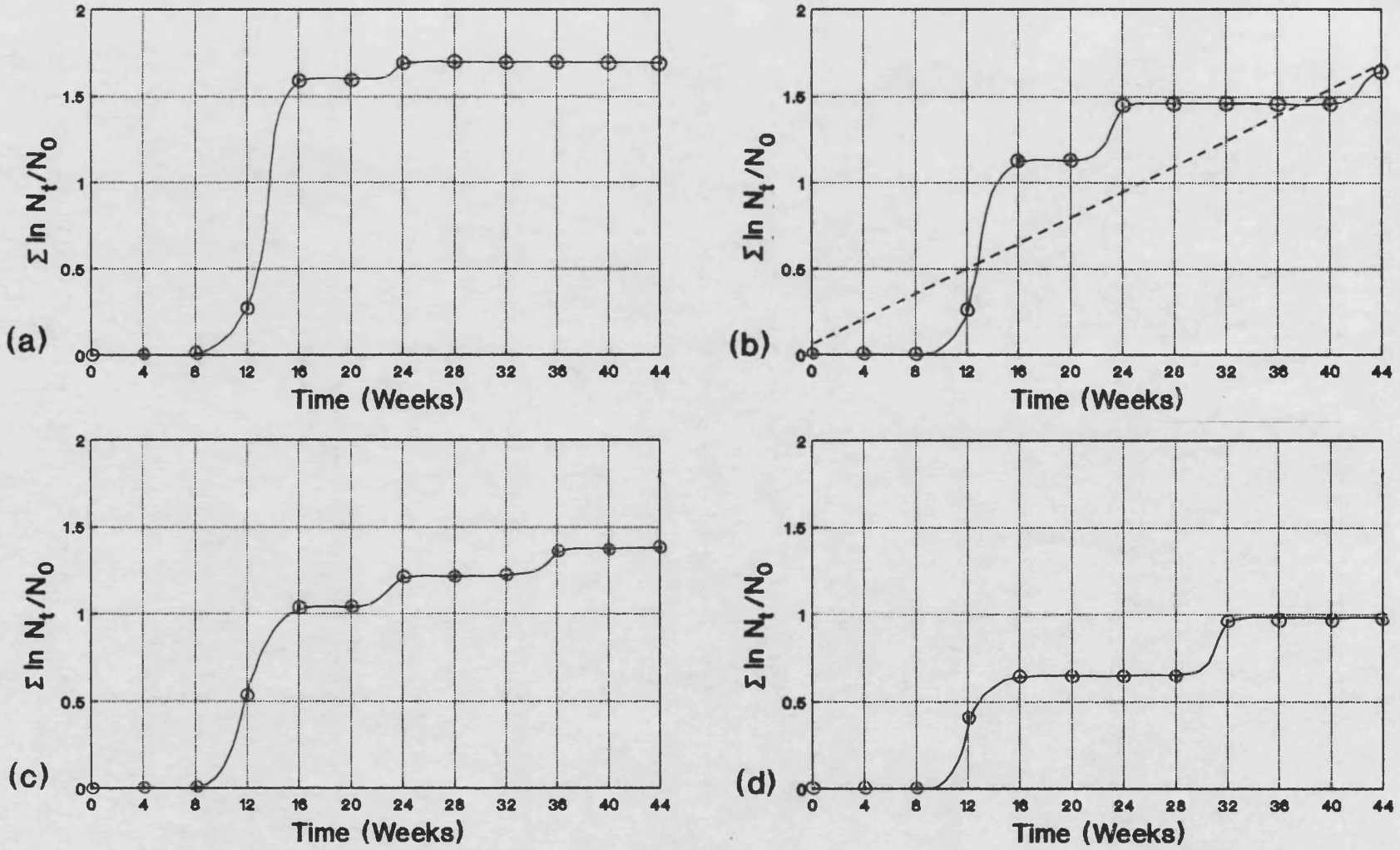


Fig 9. Shoot proliferation of nodal explants on four combinations of kinetin and BAP, (a) S-0 medium, (b) S-1 medium, (c) S-2 medium and (d) S-3 medium.

As can be seen from the Figures 9 a, b, c & d, the axillary shoot proliferation on all four media were very low. In S-0 medium, there was hardly any proliferation after 24 weeks of culture. This indicated the necessity of exogenous hormones, for the induction of axillary buds, after the primary axillary shoot growth. Some increase in shoot number was observed in S-1 medium. Both in S-2 and S-3 media, the proliferation rate decreased with time . At the end of 44 weeks, almost all of the cultures on these two media were dead leaving only 3-4 replicates. Even in media S-0 and S-1, the number of replicates were reduced dramatically after about 24 weeks of culture. Some growth was observed in the cultures on S-1 medium, even at 44 weeks of culture which was extremely slow.

The reason for the sudden increase in shoot number, between 8-16 weeks of culture, in all four media, was the subdivision of elongated primary axillary shoot into several nodes. But, the growth of the axillary buds after secondary nodes was very slow in all four media, and accordingly, the shoot proliferation rates became very low. Except in S-1 medium, no sustained shoot growth was observed. The calculated shoot doubling time for the shoots grown on this medium was 17 weeks.

(b). BAP as the only Cytokinin in the Medium.

In this experiment, BAP was the only cytokinin in the medium. NAA was used unchanged (0.2 ppm) as in the previous experiments. Nodal explants of rootstock origin were used as before. Control medium was omitted and 4 levels of BAP (2, 4, 8, and 16 ppm) were tested. M&S medium at half strength supplied with 2% sucrose was used. Media were prepared in Petri dishes for the first passage and in 100 ml jars afterwards.

Axillary bud break occurred in all 4 media, after 4 weeks of culture. Only a few cultures produced 2 axillary buds from the same axil. This was observed in the cultures grown on 4 ppm BAP containing medium. At 2 ppm BAP, 75% axillary bud break occurred. At 4 ppm this was more than 100% due to the reason that some of the nodes produced more than one axillary shoots

from one axil. Cultures grown on 8 ppm and 16 ppm media gave 93% and 63% of axillary bud break respectively. Axillary shoots produced on two lower levels of BAP carried a good leaf growth, while those produced on 8 ppm and 16 ppm BAP media, did not show any leaf growth at all.

The mean lengths of axillary shoots produced on four media are shown in Figure 10 (see also Appendix 6).

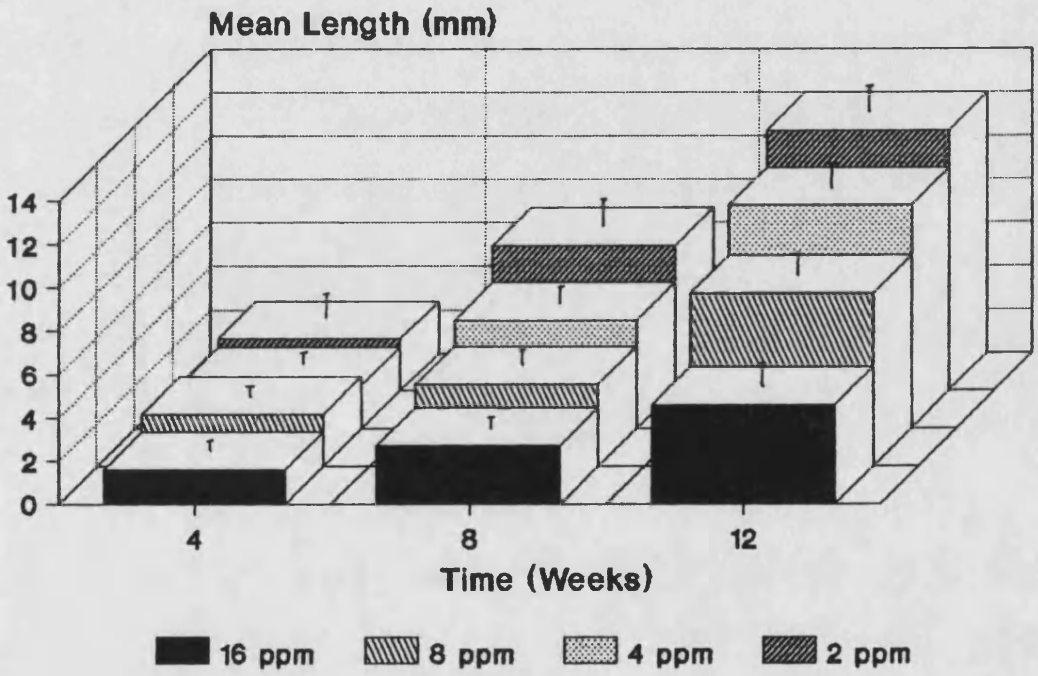


Fig 10. Mean lengths of axillary shoots of nodes at 4 levels of BAP(n=8). Cultures were recultured every 4 weeks.

It can be seen from the Figure 10, that the mean lengths of the axillary shoots decreased with increasing concentrations of BAP in the medium. Axillary shoots produced on media with 2, 4 and 8 ppm BAP were subdivided into nodes, after 12 weeks of culture. Those on 16 ppm BAP

medium did not grow long enough to divide into nodes.

After 4 weeks, secondary axillary buds were produced on secondary nodes. Growth of these buds were very slow compared to the primary nodes, as reported in earlier experiments with combinations of kinetin and BAP. Some of the primary nodes produced two axillary shoots from one axil, but this was not observed in any of the secondary nodes.

3.1.4. The Effect of Basic Medium and the Sucrose Level for Axillary Shoot Growth.

This experiment was carried out in two stages. The first was a preliminary experiment carried out with Murashige and Skoog medium and Woody Plant Medium, each with 3 levels of sucrose (2%, 4% and 8%). M&S medium was made at half strength and WPM at full strength. S-1 hormones (kinetin 2, BAP 1 and NAA 0.2 ppm) were used, and the media were designated as follows:

% of sucrose

	2%	4%	8%
WPM	S-5	S-6	S-7
MS	S-8	S-9	S-10

9 cm Petri dishes were used to make media for the first passage, and after that, 100 ml jars were used. Cultures were recultured every 4 weeks. Mean lengths of axillary shoots obtained in six media for 20 weeks of culture, are given in Figure 11 (see also Appendix 7.a).

Results to compare the basic medium, were affected by insufficient number of replicates on M&S media at all 3 levels of sucrose after 12 weeks of culture. Only 11 out of 24 cultures, survived at the end of 16 weeks. After 20 weeks there were only 9 cultures left in all 3 media (S-8, S-9 and S-10). Contrary to this, more than 80% of the cultures on WPM medium, survived

up to 52 weeks of culture. There was a significant difference between M&S and WPM media for the survival of explants. Further, the quality of the axillary shoots produced in WPM media were better than those produced on M&S media. They were thick, dark green and contained better leaves.

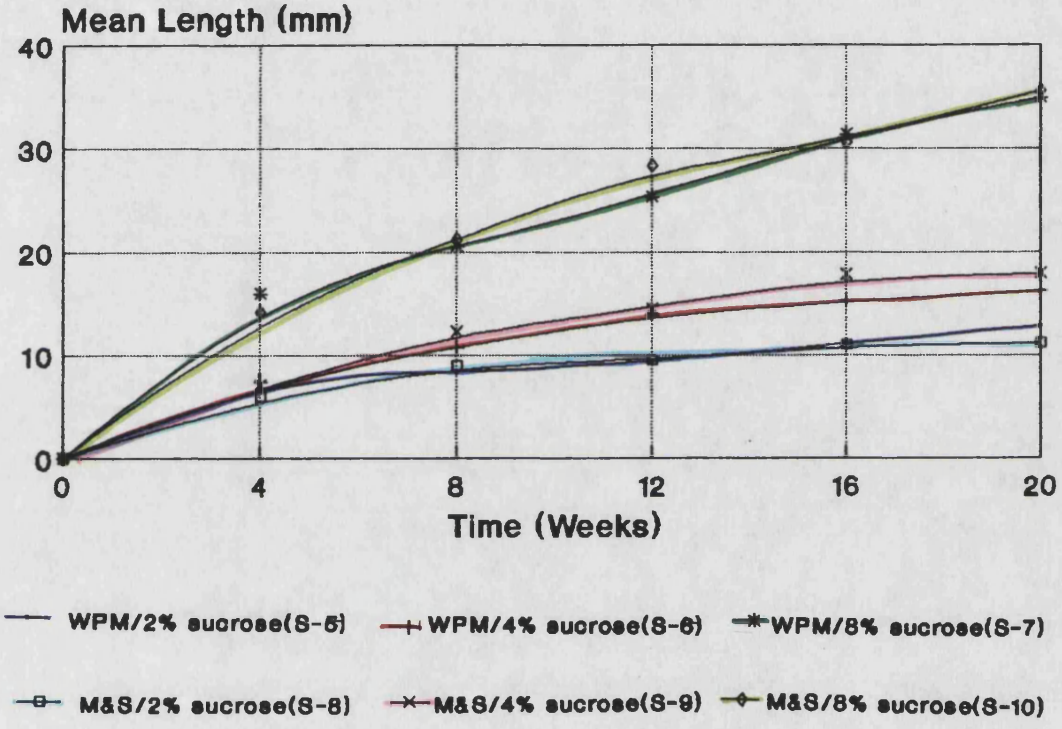
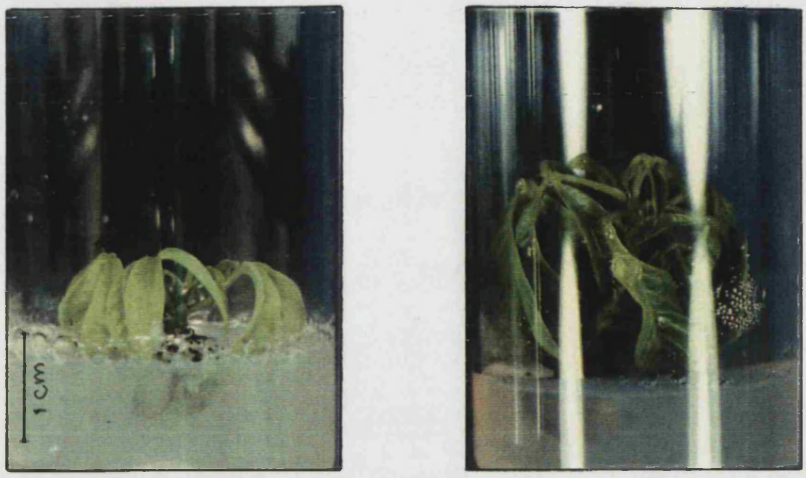


Fig 11. Mean lengths of axillary shoots of nodal explants on two basic media each at three levels of sucrose (n=8). Cultures were recultured every 4 weeks.

However, the shoot proliferation rates were calculated only for those grown on WPM media at all 3 levels of sucrose, because there was not enough replicates in M&S media after 16 weeks of culture, as stated earlier. This experiment was started with only 8 replicates. The primary axillary shoots were not subdivided into nodes until 20 weeks of culture, because a certain increase of axillary shoot length was observed at higher levels of sucrose until then. At the end of

20 weeks, the axillary shoots were subdivided into nodes. The number of axillary shoots produced, increased from 2% to 8% sucrose, because the lengths of the primary axillary shoots increased in this direction.



a. b.

Plate 4. Cultures grown on **(a).** M&S & **(b).** WPM media, at 4% sucrose. Results after 24 weeks.

Although the cultures were better grown and maintained on WPM media, still the growth rate observed with secondary and tertiary axillary shoots was low, and therefore, there was no improvement in the rate of axillary shoot proliferation. Also, as shown by the Figure 11, there was no visible effect of basic medium, on the lengths of axillary shoots.

But, the amount of sucrose in the medium was positively effective on the elongation of primary axillary shoots. The lengths of the axillary shoots increased with the increasing amount of sucrose in the medium from 2% to 8%. This was true for both WPM and M&S media separately, and in combination as well, although the number of cultures on M&S media was small after 12 weeks of culture.

This experiment was continued up to 36 weeks of culture, and the proliferation rate for the 3 levels of sucrose are given in Figure 12 (see also Appendix 7.b).

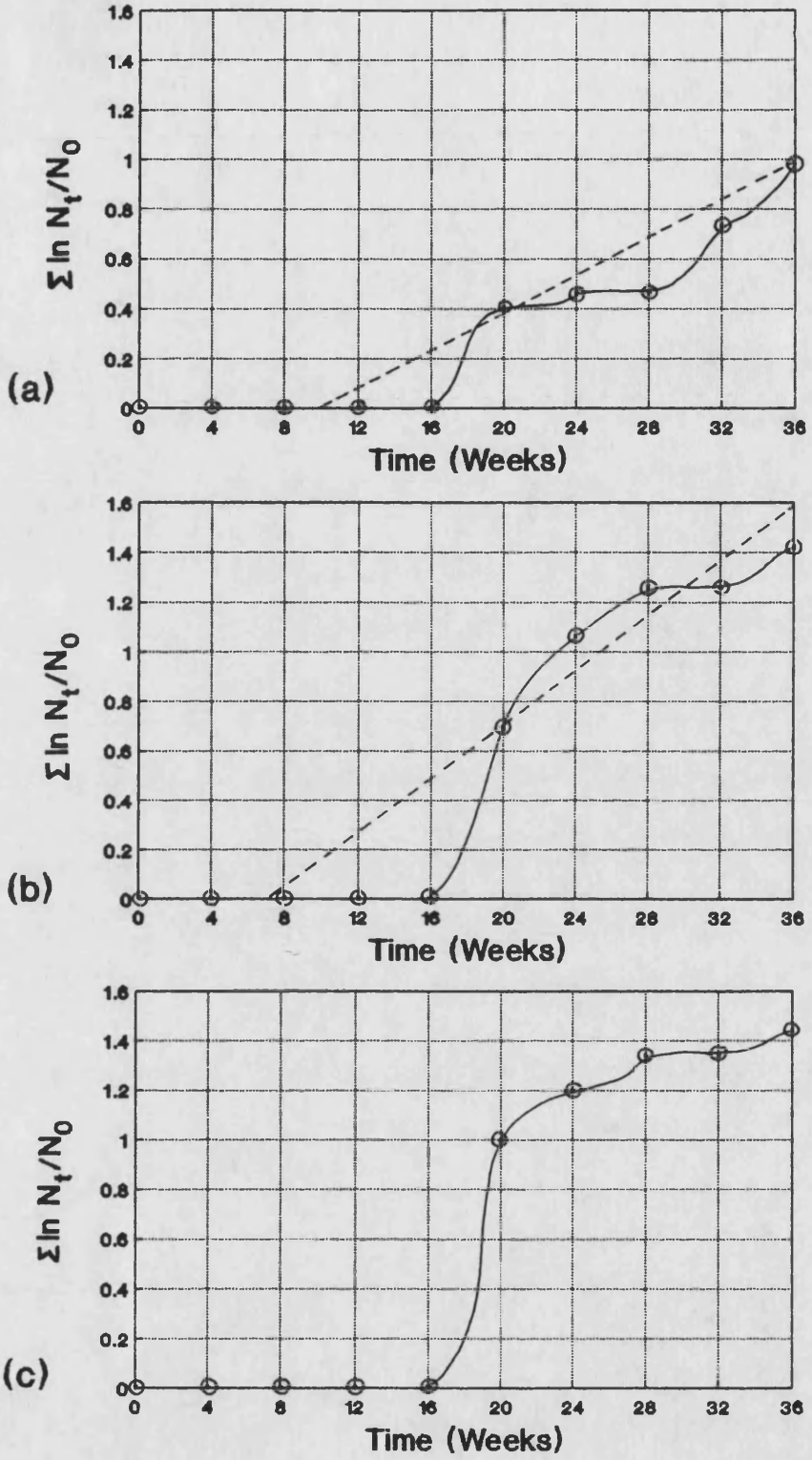


Fig 12. Shoot proliferation of nodal explants on WPM media at 3 levels of sucrose, (a) 2% sucrose, (b) 4% sucrose and (c) 8% sucrose. Cultures were recultured every 4 weeks, and first subdivision was at 20 weeks (n=8).

As the Figures 12 a, b and c show, the effect of sucrose level on the shoot proliferation rate was very difficult to be determined.

In this experiment, the first subdivision of the primary axillary shoots, was carried out only at 20 weeks of culture, causing a long lag phase. Reason for this was the continuous increase observed in the axillary shoot lengths at both 4% and 8% sucrose. The higher lengths of primary axillary shoots, however, increased the number of propagules at the first sub culture, and therefore 8% sucrose gave the highest number of propagules. But, the growth afterwards, was slow and accordingly the shoot proliferation rate became slow. Under this situation, as Figure 11 (c) shows, it was difficult to obtain a straight line to calculate the shoot doubling time.

The number of propagules produced on both 2% and 4%, were comparatively low at the first sub culture, and the proliferation afterwards was a little higher than that of 8%. As the Figures 11 (a) and (b) show, this made the calculation of shoot doubling time possible, but the culture period involved, excluding the lag phase of 20 weeks, was only 16 weeks. Therefore, it was doubtful whether the proliferation rates obtained were sustainable or not. However, shoot doubling times of 18 and 12 weeks were calculated for 2% and 4% sucrose approximately.

As far as the shoot quality and the number of propagules produced were concerned, both 4% and 8% had similar results. Shoot growth at 2% was poor compared to the other two. Therefore, 4% sucrose was chosen as the best from the three levels tested.

In the second part of this experiment, the effect of basic medium was tested again, using WPM and M&S media. But, in this experiment cytokinin type was also changed.

Sucrose was used at 4%, which was found to be the best for shoot proliferation in the previous experiment. S-1 hormones were used as the control, and thidiazuron was the new cytokinin used. Thidiazuron was tested at 0.1 ppm. NAA was present always at 0.2 ppm. Three media were prepared as follows:

1. M&S + thidiazuron(0.1 ppm)
2. WPM + thidiazuron(0.1 ppm)
3. WPM + S-1 hormones (kinetin 2, BAP 1 and NAA 0.2 ppm)

Petri dishes were used to make media for the first passage and 100 ml jars for the rest of the experiment. 8 replicates were used for each medium.

Mean lengths of axillary shoots on three media are given in Figure 13 (see also Appendix 8a).

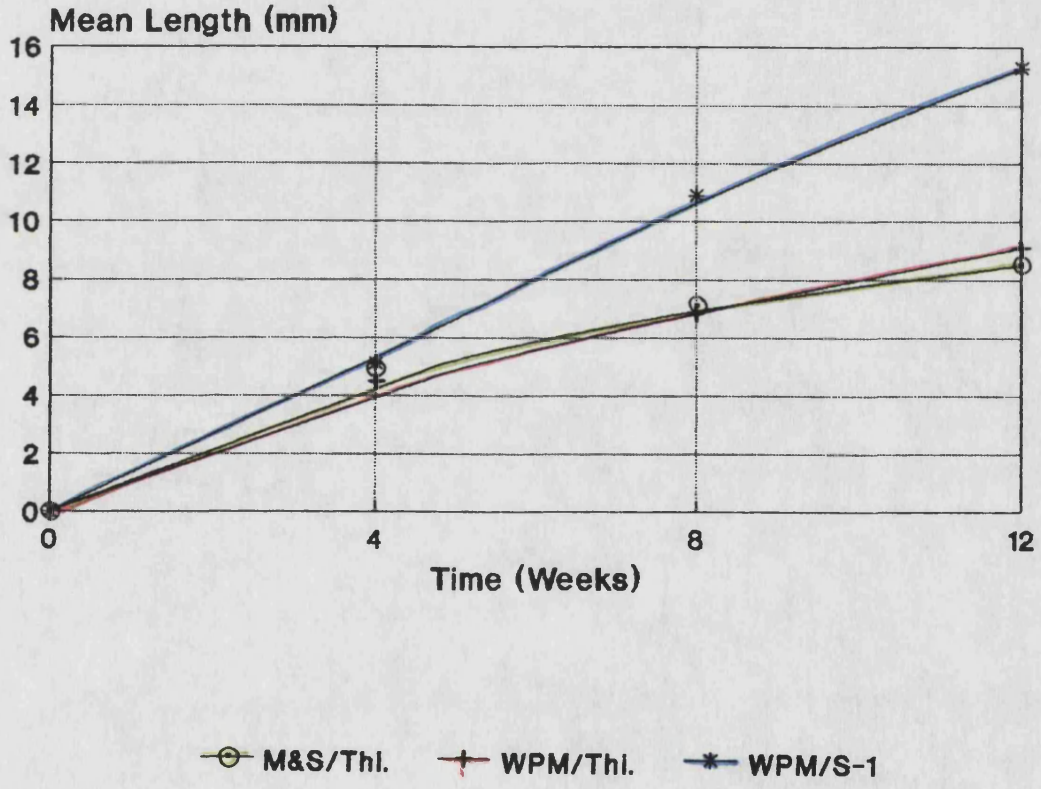


Fig 13. Mean lengths of axillary shoots of nodal explants on M&S and WPM media in the presence of thidiazuron, and a comparison of thidiazuron and conventional hormones on WPM medium. Cultures were recultured every 4 weeks. First subdivision was at 16 weeks (n=8).

Axillary shoots grown on WPM media showed a significantly better leaf growth irrespective of the growth hormones in the medium. The nodes grown on S-1 hormones, showed the maximum elongation of axillary shoots, because they produced single axillary shoots. The morphology of the axillary shoots produced on both thidiazuron containing media, were different from those produced on S-1 medium. They were very compact clusters of buds instead of single axillary shoots (Plate 5). After 16 weeks of culture, axillary shoots were divided into propagules. Those on S-1 medium were cut into nodes, while the clusters of buds produced on thidiazuron containing media, were separated into small clusters.

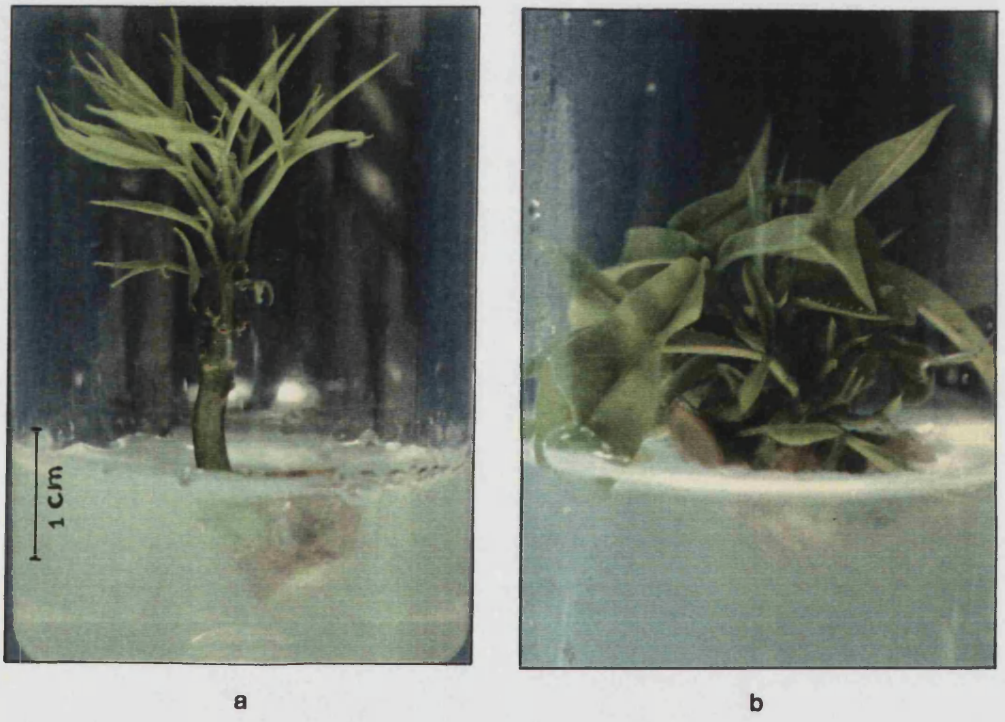


Plate 5. Morphology of the axillary shoots produced on **(a).** S-1 medium and **(b).** thidiazuron containing medium. Results at 16 weeks of culture.

Shoot proliferation up to 28 weeks of culture are shown in Figure 14(see also Appendix 8b).

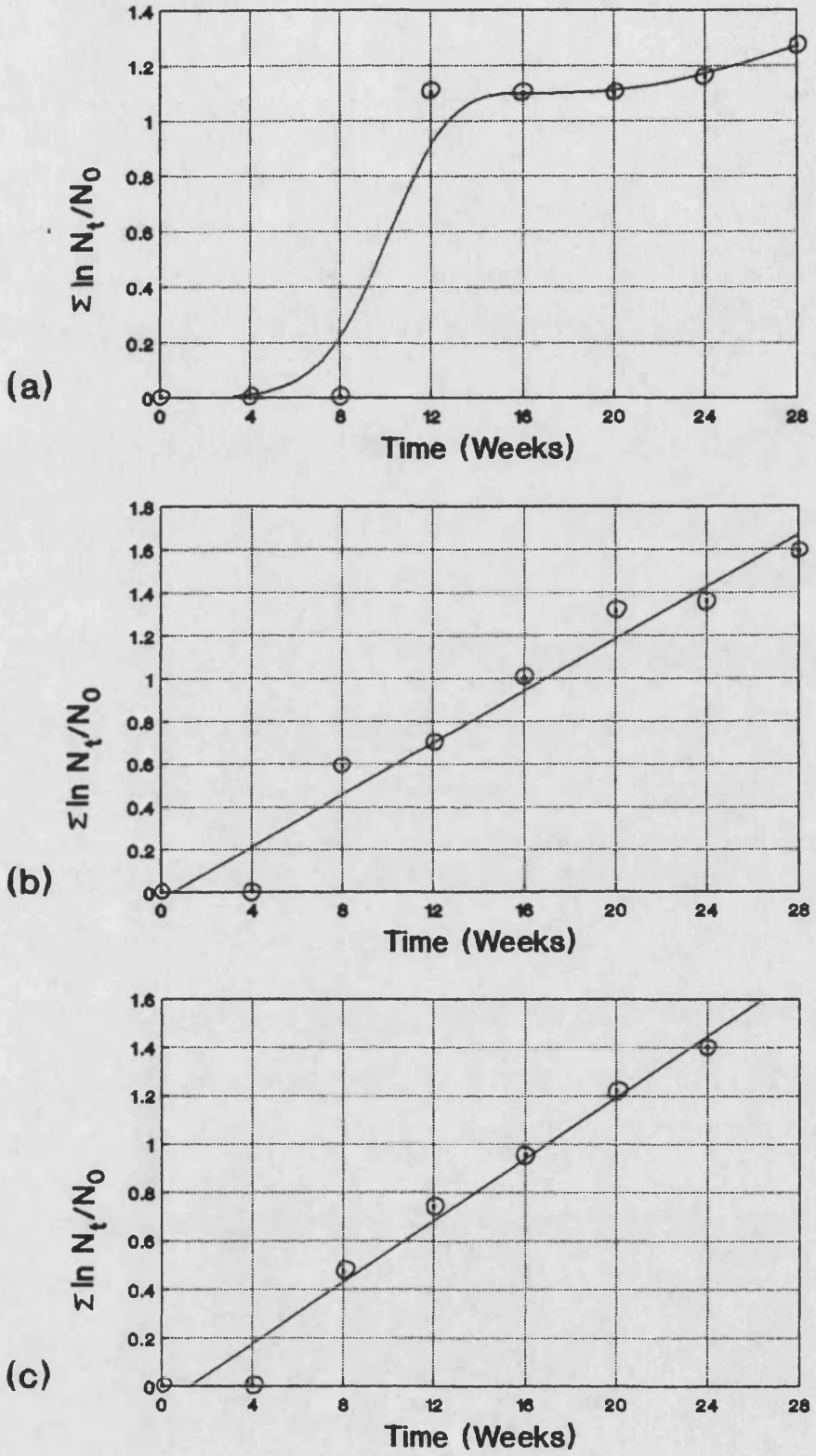


Fig 14. Shoot proliferation of nodal explants on three media (a) WPM/S-1 hormones, (b) M&S/thidiazuron and (c) WPM/thidiazuron. cultures were recultured every 4 weeks (n=8).

The shoot proliferation on 3 media were not very different, although the pattern of proliferation was different in the presence of thidiazuron. With conventional hormones, the shoot proliferation occurred in steps, because after any subculture, there was a period where the elongation of axillary buds occured. The main shortcoming in this pattern of proliferation was that the time required for this period increased gradually. This caused the rate of shoot proliferation low and therefore it was difficult to say whether the proliferation rate was sustainable or not.

In the presence of thidiazuron, the primary nodes were induced to produce multiple axillary shoots which continued in the later passages as well. Therefore, thidiazuron showed a higher proliferation rate than that of S-1 hormones, by producing multiple axillary buds instead of single shoots.

WPM medium was confirmed to be better than M&S medium for shoot proliferation, mainly because cultures did not survive on M&S medium.

After 24 weeks of culture, the number of cultures on M&S medium was reduced from 8 to 3, and all were dead after 32 weeks. All the new propagules survived in both WPM media.

The clusters of buds produced on thidiazuron media were very compact, and the concentration needed optimizing. Both S-1 hormones and thidiazuron with WPM medium gave similar shoot doubling times of about 10-11 weeks, but the cultures on M&S medium were dead at the end of 32 weeks.

3.1.5. The Effect of Thidiazuron as a Cytokinin.

The previous experiment with thidiazuron showed a better shoot proliferation pattern than with conventional hormones. This experiment was therefore carried out to confirm the results of the previous one.

A mixture of seed embryo derived and root stock derived nodal explants were used in this experiment. S-1 medium was used as the control as before. Only two levels of thidiazuron (0.1

ppm and 0.002 ppm) were tested. In both thidiazuron containing media, 0.2 ppm BAP and 0.2 ppm, NAA were also present. WPM solid media supplied with 4% sucrose, were made in 9 cm Petri dishes and were transferred into jars after the first passage. There were 8 replicates for each medium.

Shoot proliferation was calculated for each medium, and results for a period of 36 weeks are shown in Figure 15 (see also Appendix 9.a).

The media containing thidiazuron produced a higher number of axillary shoots compared to S-1 medium. Nodes on S-1 medium yielded only single axillary shoots as observed in experiments so far, which could be divided into nodes. Contrary to this, nodes on thidiazuron containing media, produced multiple axillary shoots which could be divided into 2-4 small clusters, depending on their size. Leaf growth of these was very vigorous and normal, compared to that of the shoots grown on S-1 medium.

Multiple axillary shoots produced at lower concentration of thidiazuron, were better than those produced at higher concentration. They could be subdivided easily, because the axillary shoot elongation was better. Shoot doubling times for 0.1 ppm and 0.002 ppm thidiazuron were 13 weeks and 12 weeks respectively. For S-1 medium it was 19 weeks. Unfortunately, this experiment had to be terminated prematurely due to the loss of about half of the cultures, which were overheated as a result of growth cabinet failure.

(b). Shoot Proliferation on Thidiazuron at 0.002 ppm Medium.

This experiment was carried out with the cultures survived from the previous experiment on medium containing 'thidiazuron' at 0.002 ppm. Results for a period of 68 weeks are given in Figure 16 (see also Appendix 9.b). All the cultures used in this experiment were originated from a single node.

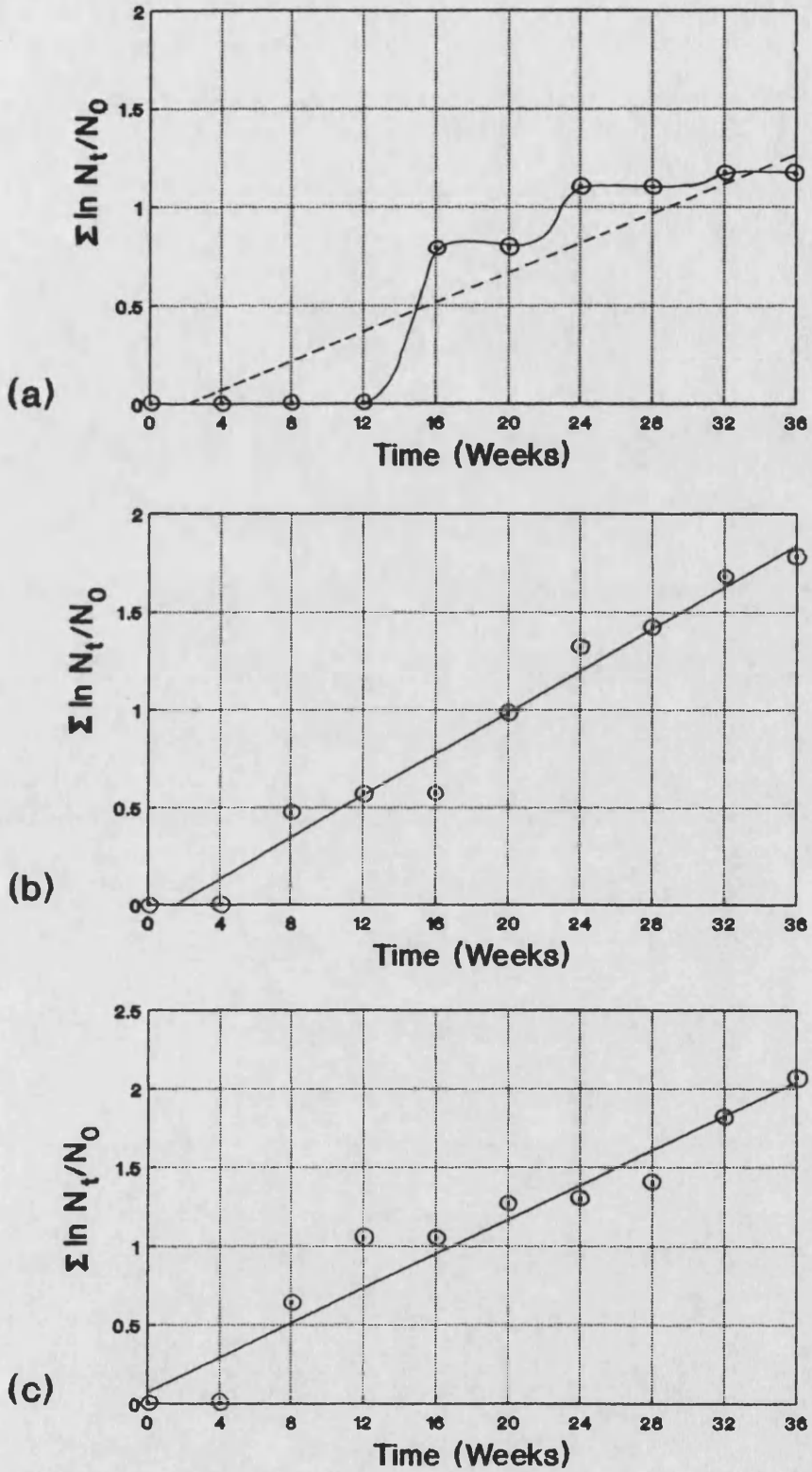


Fig 15. Shoot proliferation of nodal explants on three media, (a) S-1 medium, (b) thidiazuron 0.1 ppm and (c) thidiazuron 0.002 ppm. Cultures were recultured every 4 weeks (n=8).

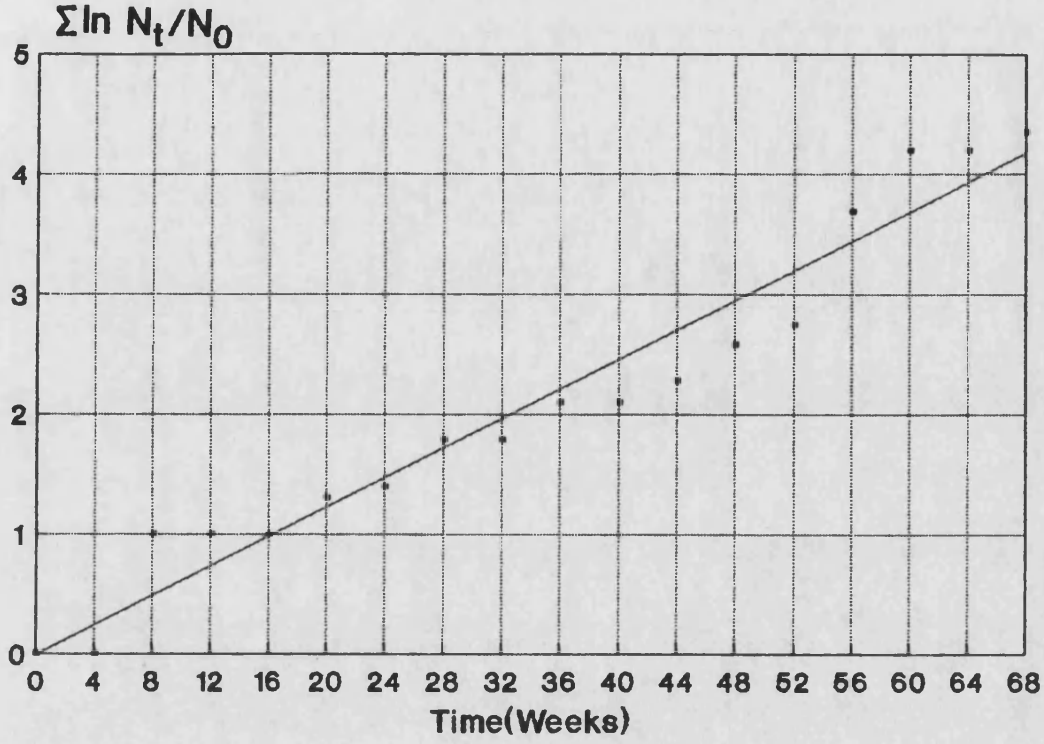


Fig 16. Shoot proliferation of a single node on a medium containing thidiazuron at 0.002 ppm. Cultures were recultured every 4 weeks while subdividing when the clusters of shoots were large enough to separate at least into two parts.

Results of this long term experiment, showed that the shoot proliferation of nodal explants could be sustained on a medium containing thidiazuron at 0.002 ppm. Shoot doubling time of 11 weeks was obtained in this experiment.

(c). Optimizing the Thidiazuron Level for Axillary Shoot Proliferation.

Explants used in this experiment were originated from 6-8 rootstock derived nodes. But, they were multiplied on 0.002 ppm thidiazuron containing medium, and were grown on hormone free medium for 6 weeks prior to use, because a large variation in growth was always observed with rootstock derived explants. Somewhat uniform explants could be obtained by growing them

in vitro over a period of time.

WPM medium supplied with 4% sucrose was used with three levels of thidiazuron (0, 0.002, 0.02 and 0.2 ppm). NAA was used at 0.2 ppm, but no BAP was used in this experiment.

Media were prepared in 100 ml jars, and the cultures were subcultured every 4 weeks. Results for a period of 12 weeks are shown in Figure 17 (see also Appendix 9.c).

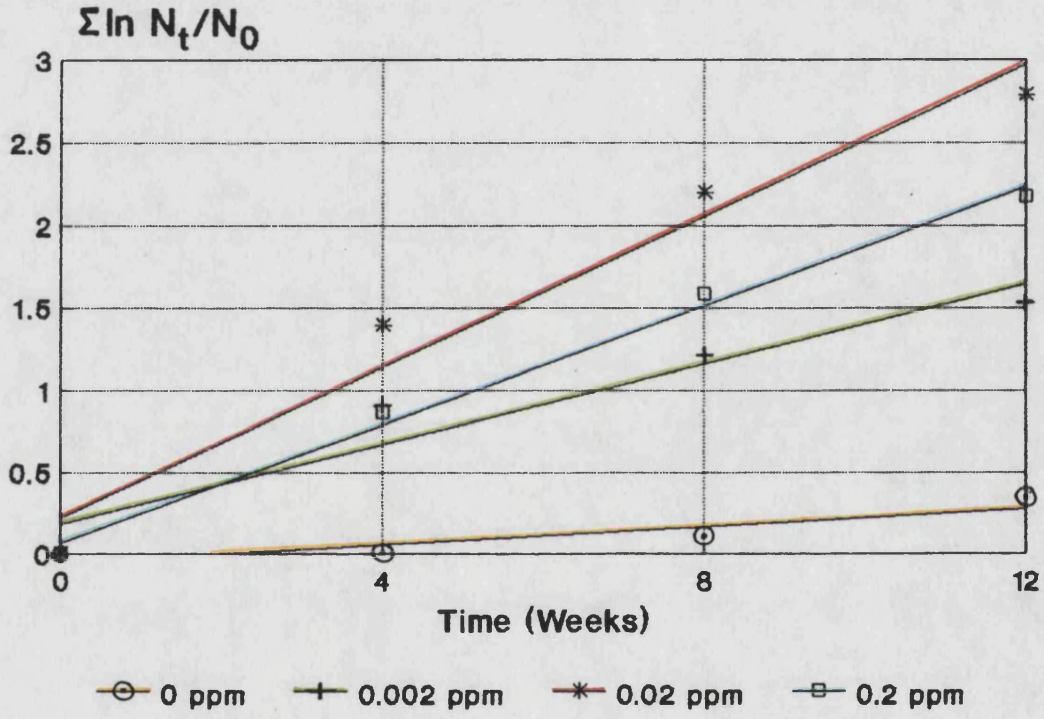


Fig 17. Shoot proliferation on thidiazuron containing media (n=10). Cultures were recultured every 4 weeks.

As can be seen from Figure 17, no lag phase was observed in this experiment, because all the explants were originated *in vitro*. They started to grow soon after their transfer onto particular media.

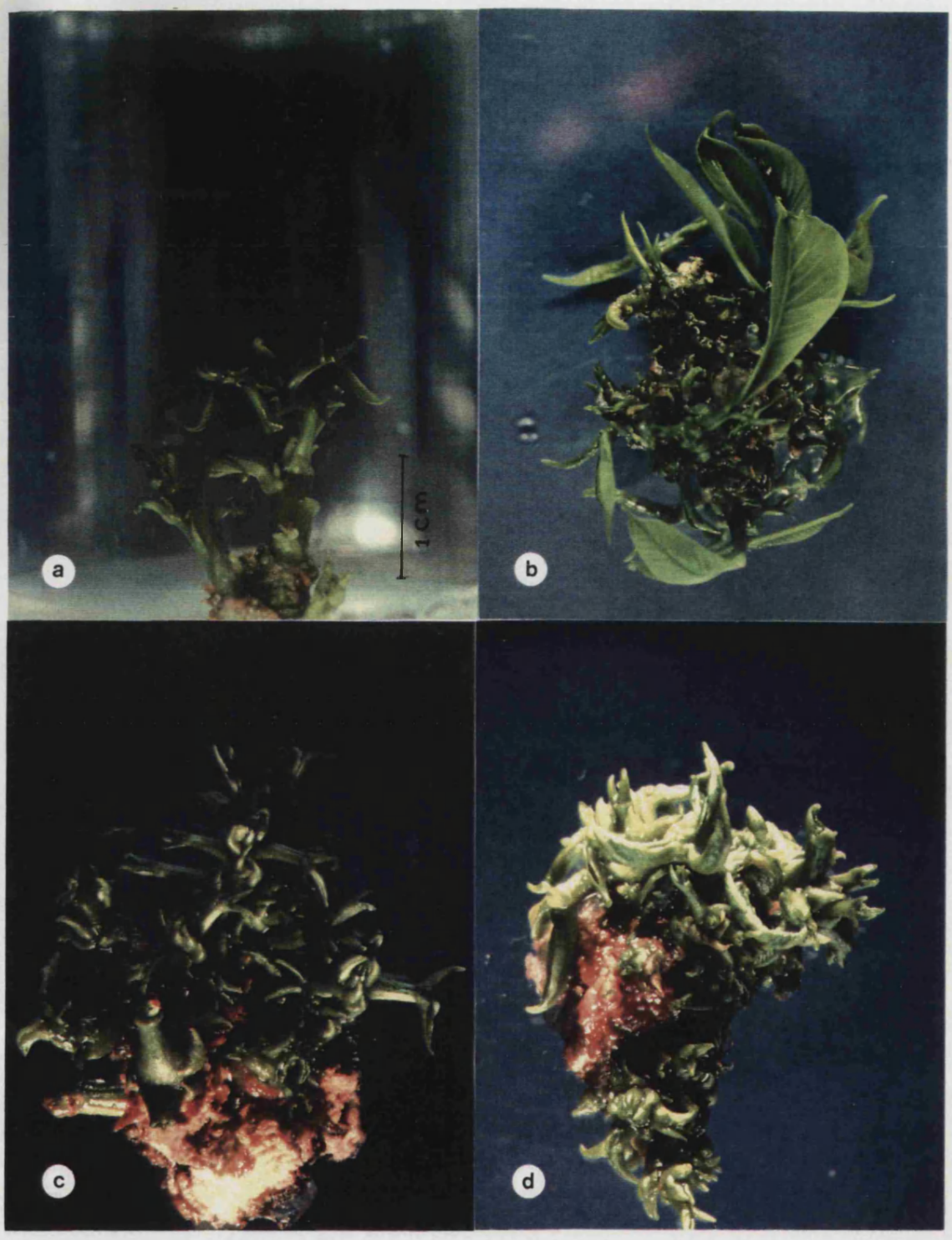


Plate 6. Shoot growth on thidiazuron containing media (a) o ppm, (b) 0.002 ppm, (c) 0.02 ppm and (d) 0.2 ppm. Results after 12 weeks.

After 4 weeks of culture, more than 60% of the cultures on control medium gave only one axillary shoot, while the rest of them gave two axillary shoots. The shoots produced were 5-20 mm size and contained normal leaves. No multiple axillary shoot formation was observed in any of the cultures on control medium (Plate 6.a). Roots were formed on about 40% of the cultures at 12 weeks. Shoot doubling time for the cultures grown on control medium was about 33 weeks.

Multiple axillary shoot formation with normal leaves was observed in 0.002 ppm thidiazuron medium (Plate 6.b). Axillary shoots produced were 5-15 mm in height. 25% of the cultures grown on this medium, produced roots after 12 weeks. Shoot doubling time was 39 days in this medium. Root formation was not observed in the previous experiment with 0.002 ppm thidiazuron; that medium contained BAP at 0.2 ppm in addition to thidiazuron.

Thidiazuron at 0.02 ppm showed the maximum proliferation; the shoot doubling time was only 23 days. Axillary shoots produced were 5-10 mm in size, and contained a very good leaf growth. No root formation was observed on any of the cultures in this medium.

The shoot doubling time for the cultures grown on 0.2 ppm thidiazuron medium was 28 days. But, the propagules produced in this medium had no proper axillary shoots as observed on the other 2 media. They were only clusters of buds which could be divided into small clusters (Plate 6.d). Leaf growth on this medium was poor compared to all other media. No root formation was observed on any of the cultures. Attempts were made to induce the clusters of buds grown on this medium (thidiazuron 0.2 ppm), to produce normal axillary shoots by transferring them onto a medium containing only 0.002 ppm thidiazuron. After 4 weeks on low thidiazuron containing medium, the propagules still produced clusters of buds. But after 8 weeks of culture on the same medium, axillary shoots of 5-10 mm size were produced with normal leaves.

The single axillary shoots produced on control medium, were also tested for their ability to produce multiple axillary shoots in the presence of thidiazuron. They were transferred onto 0.02 ppm thidiazuron medium, and multiple axillary shoots were observed after 4 weeks of culture.

3.1.6. Selection of Nodal Explants.

(a). The Effect of the Growth Stage of the Shoot, from which the Nodes were Harvested.

The normal growth pattern of a free growing shoot *in vivo*, was as in Figure 18 (see also Appendix 10).

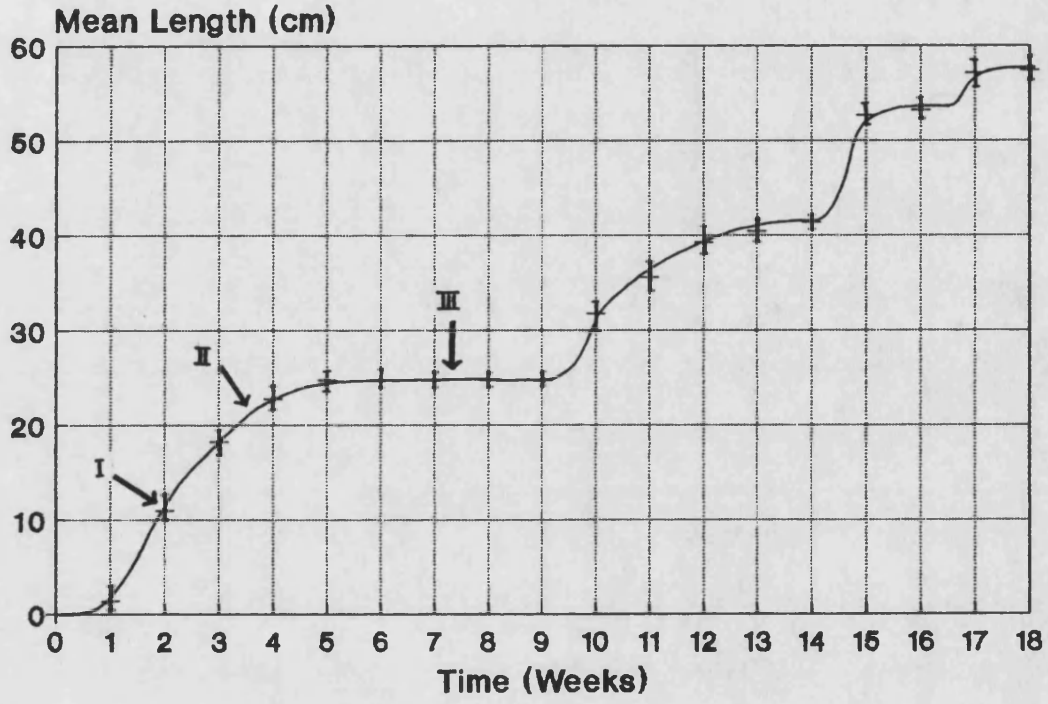


Fig 18. The growth pattern of a free growing shoot *in vivo* (n=10).

As shown in Figure 18, in a free growing shoot, 3 main stages of growth can be identified:

(I). Actively growing phase; very young shoots at their copper brown stage with no expanded leaves. The mean length of the whole shoot was about 9 cm.

(II). Intermediate phase; young shoots with copper brown stems, but contained expanded leaves; leaves too were copper brown. The mean length of whole shoots was about 20 cm.

(III). Stationary state of growth; mature green woody stems with green leaves. Average length of whole shoots was 25 cm.

Nodes were taken from the three stages mentioned above, and cultured onto a hormone free medium in Petri dishes. Solid WPM medium with 4% sucrose was used. There were 8-16 replicates from each type.

Mean lengths of axillary shoots produced, for a period of 16 weeks are given in Figure 19 (see also Appendix 11a).

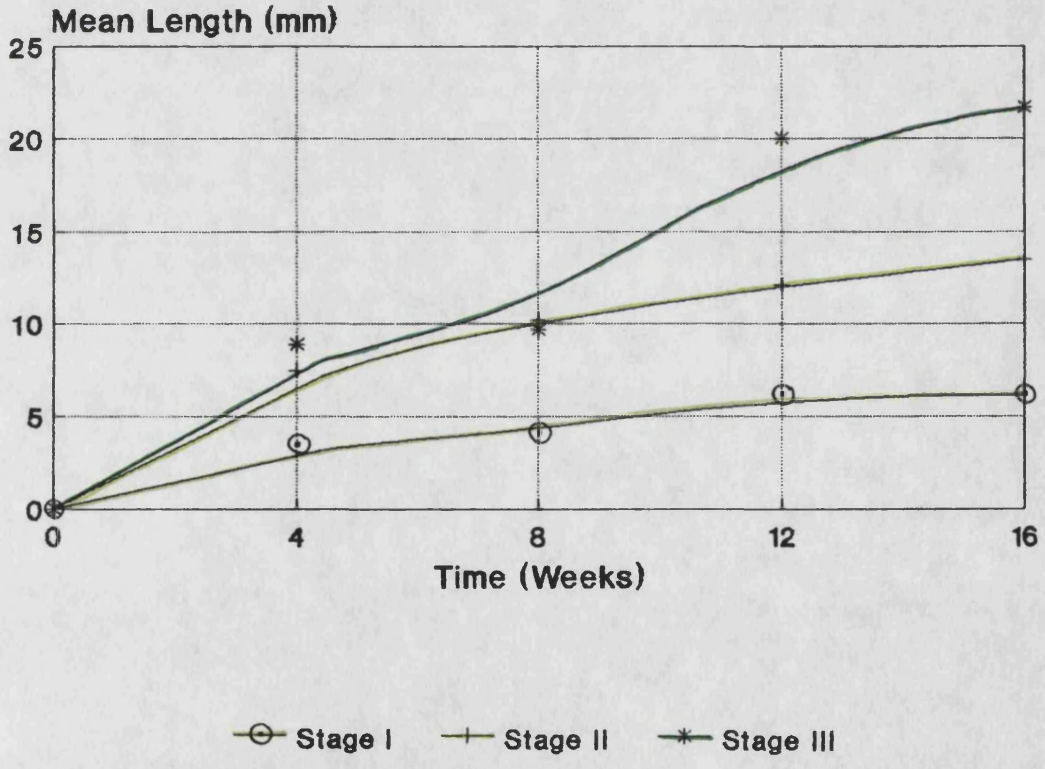


Fig 19. Mean lengths of axillary shoots of nodes harvested at three stages of growth (n=8-16)

As Figure 19 shows, the maximum elongation of axillary shoots was observed with type III nodes originated from more mature shoots. The nodes removed from very young shoots at actively growing phase (type I), showed the least elongation of axillary shoots while the nodes of type III had the best elongation. Roots were produced on about 25% of the culture of type II and III at 16 weeks of culture.

After 16 weeks of culture, axillary shoots which were more than 1 cm long, were cut into nodes and transferred into a medium containing thidiazuron at 0.002 ppm. No proliferation was observed of the nodes from stage I and Stage II for another 8 weeks, but slight proliferation was observed from the nodes of stage III. Number of propagules produced per explant up to a total of 32 weeks are shown in Figure 20 (see also Appendix 11.b).

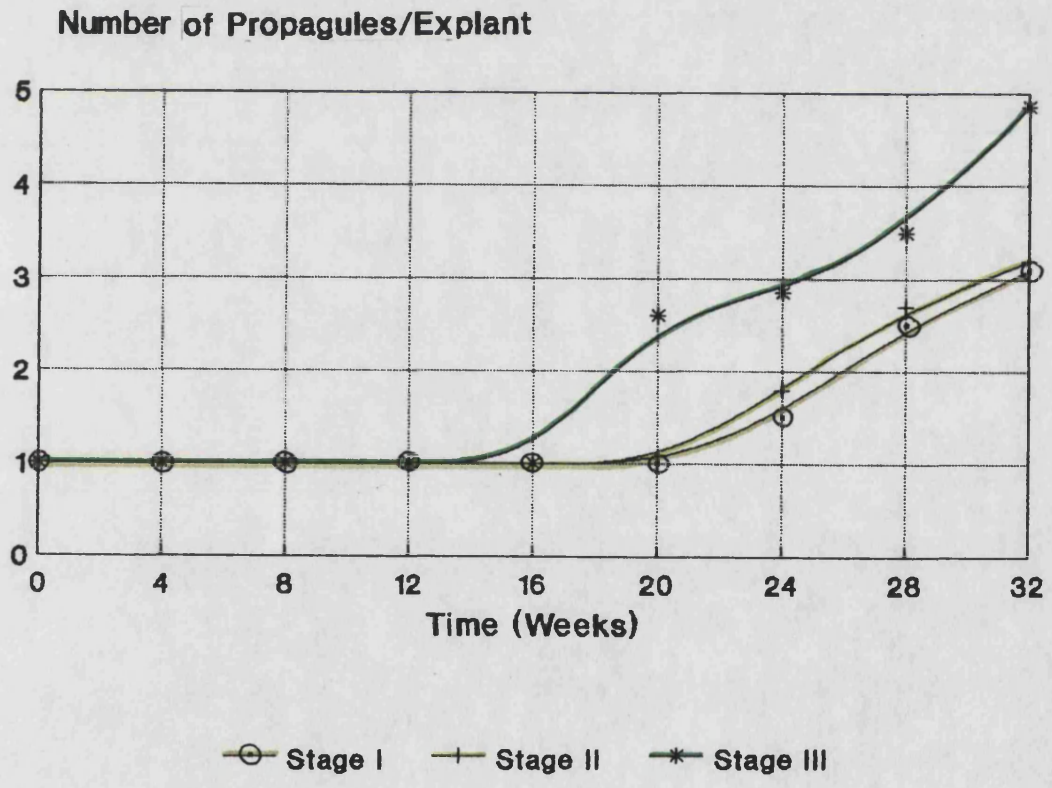


Fig 20. Number of propagules per explant of nodes harvested at three stages of growth(n=8-16).

Multiple axillary shoots were produced on newly formed nodes, on their transfer to thidiazuron containing media (at 0.002 ppm). The growth of these secondary nodes, was slow compared to that of primary nodes observed in previous experiments in the presence of thidiazuron. The growth of the multiple axillary shoots originated from stage III shoots, were better than those originated from the other two types.

(b). The Effect of the Position of the Node (in the Shoot) on Axillary Shoot Production.

In this experiment, shoots of 15-20 cm were removed from the stock plants. All leaves were removed and nodes were separated starting from the top of the shoot, and collected into numbered jars from 0-7, 0 being the shoot tip (Figure 21).

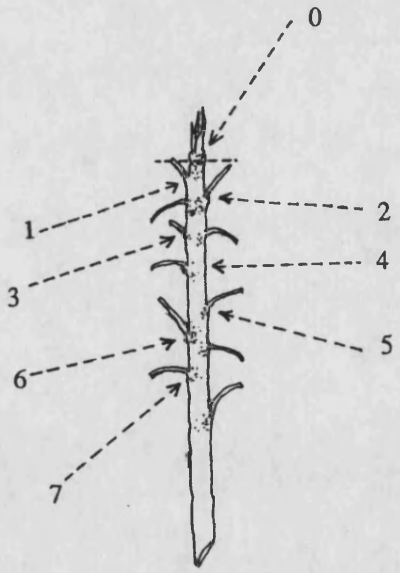


Fig 21. Nodes numbered from 0 to 7 according to their position on the shoot.

Node size varied from 2 to 5 cm and there were 11 replicates from each type. Nodes were cultured onto a hormone free, solid WPM medium supplied with 4% sucrose. Mean lengths of the primary axillary shoots produced, are given in Figure 22 (see also Appendix 12.a).

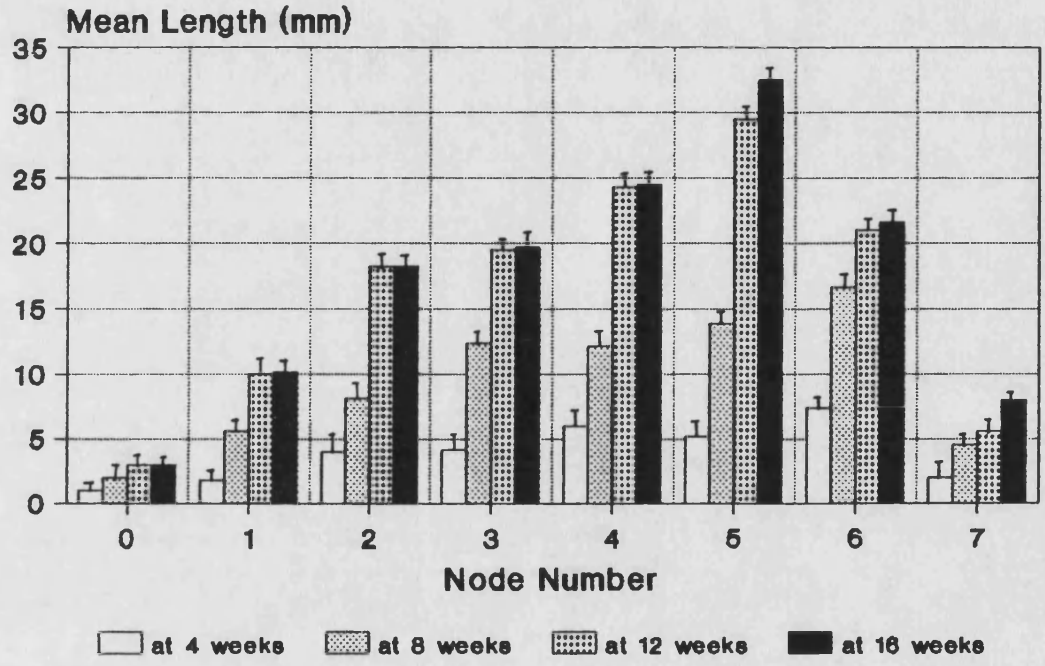


Fig 22. Mean lengths of axillary shoots of nodal explants, numbered from 0 to 7, according to their position (n=11).

As can be expected, non of the axillary buds on shoot tips grew beyond 2-3 mm. The mean lengths of the axillary shoots increased from the shoot tip up to the node number 5, and started to decrease. Roots were produced on about 25% of the shoots at 16 weeks of culture, irrespective of their position. At this stage, axillary shoots were divided into nodes, and cultured onto S-1 medium (kinetin 2 ppm, BAP 1 ppm and NAA 0.2 ppm).

The number of propagules produced by each type of node, at 16 weeks, was as in Figure 23 (see also Appendix 12.b).

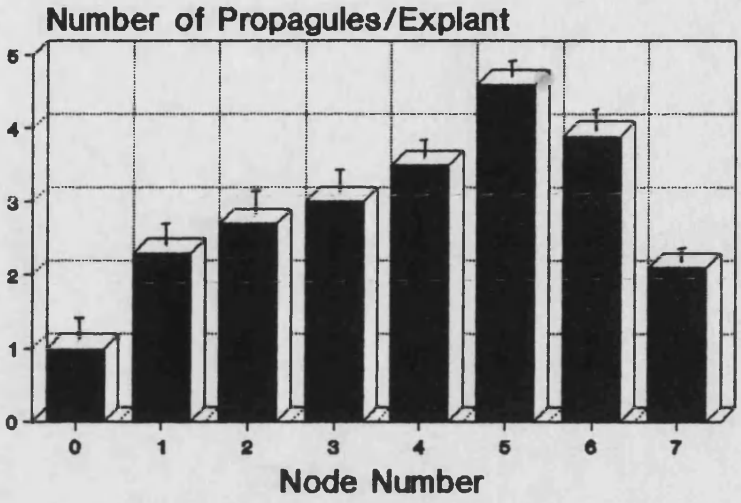


Fig 23. Number of propagules produced by nodes, numbered from 0 to 7 according to their position, Results at 16 weeks (n=11).

As can be seen from the Fig 23, the number of propagules produced increased from the shoot tip up to the node number 5, and then started to decrease. The number of propagules produced by the nodes numbered from 0 to 7 were 1, 2.3, 2.7, 3, 3.5, 4.6, 3.9 and 2.1 respectively. These figures were directly related to the lengths of primary axillary shoots. The growth of the secondary axillary shoots were again very slow, and therefore, the situation remained unchanged for another 12 weeks, indicating that the position of the node was mainly effective on the growth of primary shoot.

This experiment was later repeated, and the same results were obtained for the lengths of primary axillary shoots. But, this time, the secondary nodes were transferred onto a medium containing thidiazuron at 0.002 ppm. They produced multiple axillary shoots, but the growth was not as fast as that of primary nodes, as observed before. Therefore, when 'thidiazuron' is used to induce multiple axillary shoots, it is advisable to start with primary nodes which contain the highest potential to grow.

3.1.7. The Effect of GA_3 and Rooting on Secondary and Tertiary Axillary Bud Elongation.

When conventional hormones were used to induce axillary shoot proliferation, the main problem observed in the present studies was, that the growth of the axillary shoots became weaker and slower with the culture passage.

Exogenous cytokinins were not important for the growth of primary axillary shoots, and a good axillary shoot growth of about 30-50 mm, could be obtained in 8-12 weeks of culture (Plate 7.a).

For the growth of secondary axillary shoots, the presence of exogenous cytokinins was necessary, and the axillary shoot growth was very slow compared to that of primary axillary shoots. It took about 20-24 weeks for the growth of the secondary axillary shoots. The internodal lengths were small, and the thickness of the axillary shoots too was small (Plate 7.b). This situation was more pronounced with the growth of tertiary axillary shoot (Plate 7.c).

The aim of this experiment was to see whether this situation could be improved by the use of GA_3 in the culture medium or by inducing roots on the axillary shoots prior to harvest nodal explants from them.

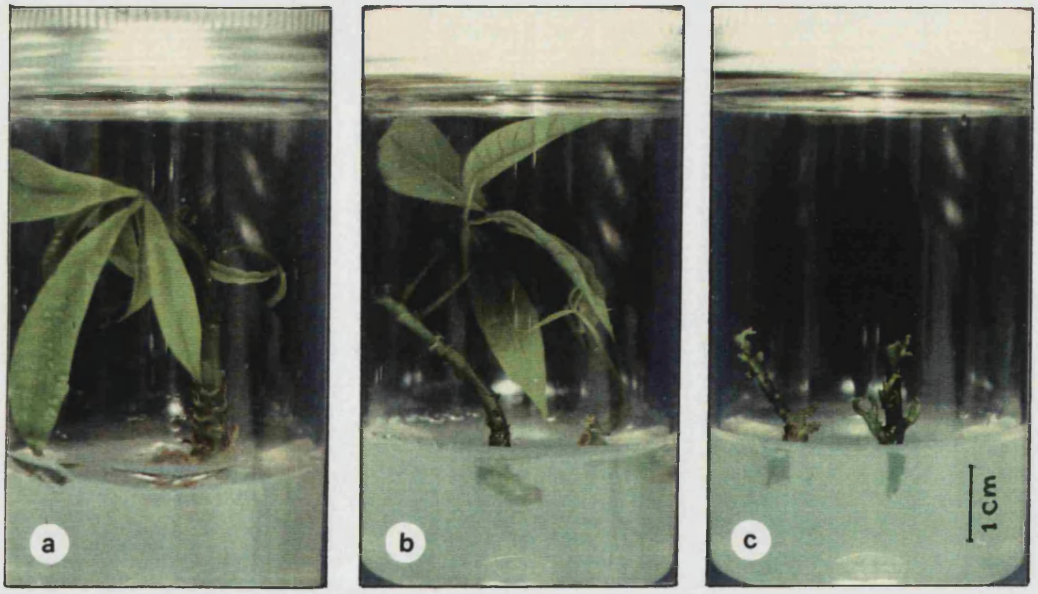


Plate 7. Axillary shoot growth of (a) Primary, (b) Secondary and (c) Tertiary nodes on S-1 medium.

(a). The Effect of GA_3 .

Only secondary and tertiary nodes were used in this experiment. They were grown on hormone free medium for 4 weeks prior to use.

Solid WPM medium supplied with 4% sucrose, was made with 0, 0.5, 1, 2, 4 and 8 ppm GA_3. 10 explants were used in each medium, and cultures were recultured onto fresh media every 4 weeks.

After 4 weeks of culture, no difference was observed between media, and no elongation was seen in any of the cultures. Good leaf growth was observed on 1 ppm GA_3 containing medium. At the end of 8 weeks, a little apical elongation was observed in only one culture at 8 ppm GA_3 medium.

Therefore, one more higher level of GA_3 (16 ppm) was introduced, and 0.5 ppm level was omitted after 12 weeks of culture. Also, media were supplied with S-1 hormones (kinetin 2 ppm, BAP 1 ppm and NAA 0.2 ppm), in addition to GA_3.

After 16 weeks of culture, nodes on 1 ppm GA_3 produced good leaves, and about 30% of the cultures showed axillary bud elongation of about 1 cm.

After 20 weeks of culture, about 40% of the nodes on 16 ppm GA_3 medium showed axillary bud elongation of 3-5 mm. Leaf growth was observed in some cultures in all media. But, no further elongation of axillary buds was observed even after 24-28 weeks of culture.

The effect of GA_3 was tested with clusters of buds produced on 0.02 ppm thidiazuron medium. Axillary shoots were 3-5 mm long when they were transferred onto 16 ppm GA_3 medium. After 8 weeks on GA_3 containing medium, 20-30 mm axillary shoot elongation was observed in all the clusters of buds. They all carried good leaves. The medium used, contained no other growth hormones, but GA_3. Control medium gave only 10-15 mm elongation of axillary shoots. They all carried good leaves. Although the elongation of multiple axillary shoots produced on thidiazuron containing media, was satisfactory, this could be accelerated by using

GA_3 in the medium. The concentration of GA_3 may need to be optimized, because only one level was tested in this experiment.

(b). The Effect of Rooting .

Secondary shoot tips and nodes grown on S-1 medium were used in this experiment. Two rooting media were tested, and S-1 medium was used as the control. One rooting medium contained 2 ppm IBA (RM 4 medium), and the other medium contained 0.5% activated charcoal, in addition to the IBA (RM 5 medium). These two media were found to produce substantial roots on shoot explants in experiments carried out for rooting of shoot tips. Half strength solid WPM medium supplied with 4% sucrose, was used in rooting media. For S-1 medium, WPM was used at full strength with 4% sucrose. All three media were made in tubes, and there were 10 nodes and 10 shoot tips for each medium. Cultures were recultured onto fresh media every 4 weeks.

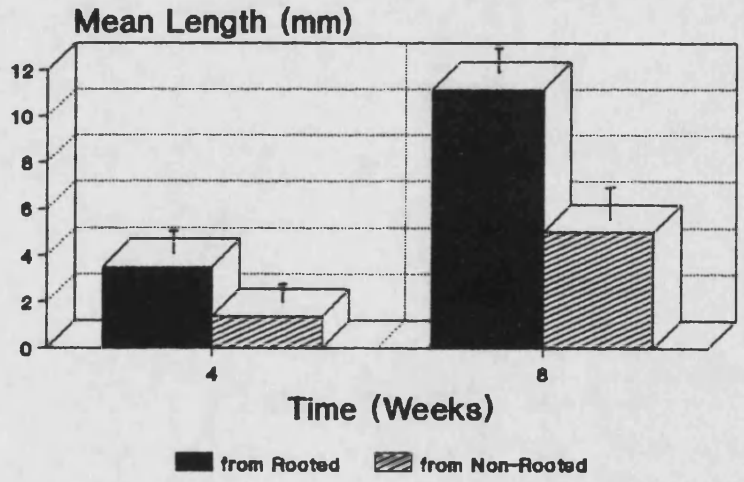


Fig 24. Axillary shoot elongation of secondary and tertiary nodes, derived from rooted and non-rooted cultures (n=15).

After 8 weeks of culture, 60% of the nodes and shoot tips, on RM 4 medium and 30% of them on RM 5 medium produced roots. No root formation was observed on the cultures on S-1 medium. Cultures on all three media produced leaves. But the leaf growth in RM 5 medium which contained charcoal, was significantly better.

After 12 weeks of culture, nodes were removed from the rooted cultures and from the cultures on S-1 medium. They were all cultured onto S-1 medium. There were 15 replicates from each type (from rooted cultures and from non rooted cultures).

Results after 8 weeks on S-1 medium are shown in Figure 24 (see also Appendix 13). As it can be seen, there was a significant difference between the two origins, in the lengths of new axillary shoots produced.

3.1.8. Rooting of Shoot Tips.

In this experiment, 1-3 cm long shoot tips harvested from *in vitro* grown plants were used. Half M&S solid medium supplied with 4% sucrose, was used with a combination of IBA and charcoal as follows:

IBA (ppm) \ % of charcoal	0	0.5	5.0
0	RM 1	RM 2	RM 3
2	RM 4	RM 5	RM 6

Roots were formed within 4-8 weeks, in all 6 media. But, the roots formed on RM 4 medium, were significantly better in quality than those on other media; they were fast growing, strong and contained lateral roots (Plate 8). The leaf growth of shoots grown on charcoal containing media, were better and dark green always.

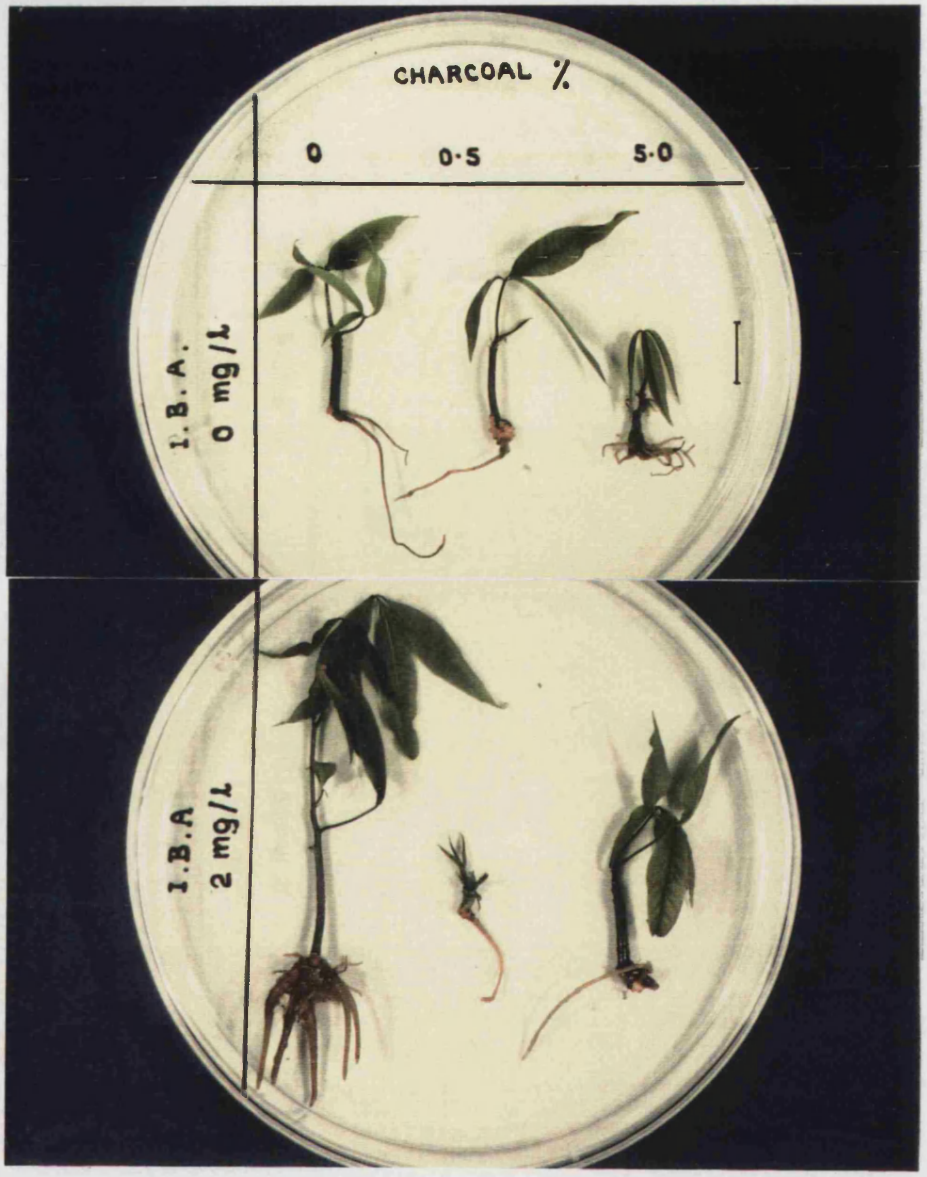


Plate 8. Root formation on shoot tip explants in six rooting media. Results at 12 weeks of culture.

Results up to 16 weeks of culture are given in Figure 25 (see also Appendix 14) and Table 4.

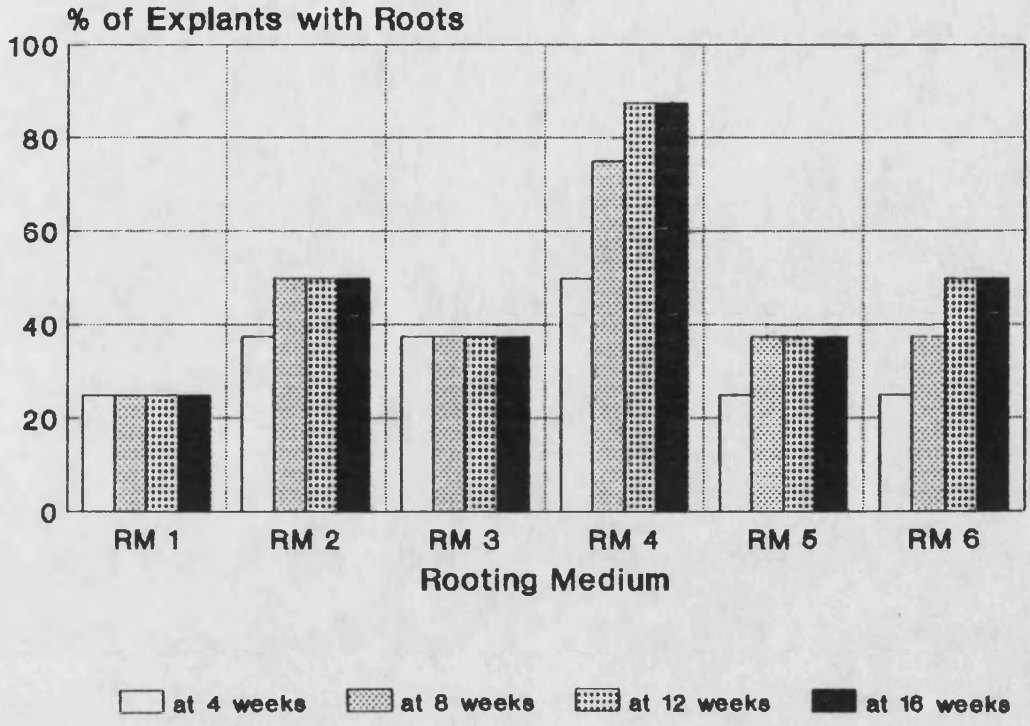


Fig 25. Root formation of seed embryo derived shoot tips on 6 rooting media (n=8).

	RM 1	RM 2	RM 3	RM 4	RM 5	RM 6
% of rooting	25	50	38	87	38	50
Mean length of roots (mm)	36	23	20	21	18	27
Approximate diameter (mm)	1mm	1mm	1mm	1mm	1mm	1mm
Presence of lateral roots	-	-	-	+	-	-
Mean growth of the shoot(mm)	2.2	1.2	10	15	17	18
Leaf growth	poor	good	good	good	good	good

Table 4. Root formation on shoot tip explants on six rooting media. Results after 8 weeks (n=8).

Since the roots produced on RM 4 medium, were found to be better than those on the other media, another set of seed embryo derived shoot tips were cultured onto RM 4 medium.

Shoot tips, 2.5-4 cm long, with and without leaves (defoliated before culturing), were used in this experiment. Results after 8 weeks are shown in Table 5.

Roots were initiated on 80% of the shoot tips within 3 weeks of culture. After 7 weeks, 90% of shoot tips produced roots, similar to those on RM 4 medium in the previous experiment; roots produced were strong, fast growing and contained lateral roots.

No difference was observed between the roots produced on shoot tips with leaves and without leaves. Most of the cultures produced good leaves.

	with leaves	without leaves	mean
% of rooting	100	80	90
Mean length of roots(mm)	17	21	19
Mean no.of roots per shoot	6.4	3	4.7
Mean length of shoot growth	15	9	12
Leaf growth	good	good	good

Table 5. Root formation on leafy and defoliated shoot tips on RM 4 medium, results after 8 weeks (n=10).

Rootstock derived shoot tips were also tested for their ability to produce roots. In this experiment a mixture of secondary and tertiary shoot tips of 2-3 cm long, were used. There were 8 replicates.

After 8 weeks of culture, 100% rooting was observed on both secondary and tertiary shoot tips. They were similar to those produced by seed embryo derived shoot tips.

The effect of the size of the shoot tips on rooting, was also tested with rootstock derived materials. Shoot tips of 0.5, 1.0, 1.5, 2.0 and 2.5 cm long were cultured onto RM 4 medium.

After 8 weeks of culture, roots were initiated in all the cultures. No difference was observed between the roots produced on different sizes of shoot tips (Plate 9). But, no apical elongation or leaf growth, was observed on shoot tips of 0.5 cm size, but all other sizes showed apical elongation of 10-30 mm with good leaf growth.

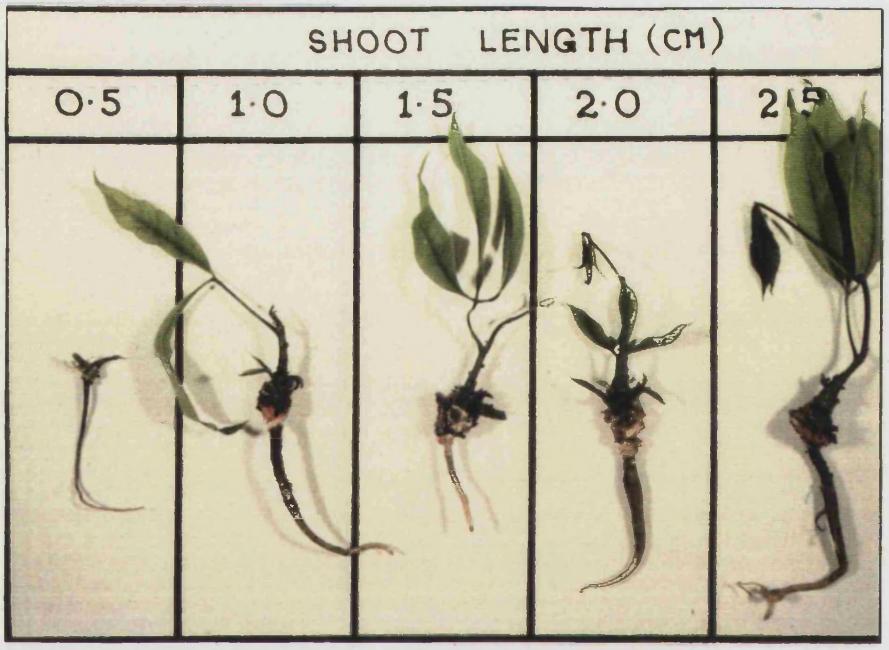


Plate 9. The effect of explant size on rooting. Results at 12 weeks of culture.

Plate 10. Rooting on axillary shoots produced on thidiazuron containing media.

The multiple axillary shoots (or clusters of buds) produced on thidiazuron containing media, were also tested for their ability to produce roots.

In this experiment, 10 cultures each containing only one axillary shoot of about 0.5-1.0 cm size, were cultured onto RM 4 medium in jars. After 6 weeks of culture, all the cultures produced strong roots containing lateral roots (Plate 10). Good leaf growth was observed on elongated apices after about 12 weeks.

3.1.9 Weaning of Plantlets.

Weaning of the rooted plantlets, was carried out mostly with the shoots produced with thidiazuron.

After about 12-16 weeks on rooting medium, plantlets were taken out and washed thoroughly under running tap water. They were then transferred onto M-4 potting mixture in small propagation trays, where high humidity was maintained for the first two weeks. After about 12-16 weeks, apical elongation was observed.

3.2. Discussion

The results presented in the study with juvenile material of *Hevea*, showed that the nodes were more responsive and productive explants in the production of axillary shoots, compared to that of shoot tip explants.

Axillary buds produced on shoot tips, did not elongate substantially and therefore, the explants remained as shoot tips with no axillary shoot growth(Plate 2b on page 53). Roots could be induced on these shoot tips by transferring them onto a cytokinin free medium, or in the presence of IBA at 2 ppm.

Although this behaviour of shoot tips has not been mentioned by those who worked only with nodes, it is supported by the fact that there has been no report published so far, on successful propagation of *Hevea* by shoot tip culture. The only reports available on shoot tip culture of *Hevea*, were the root formation on 2-3 mm long *in vitro* grown apices, done by Paranjothi and Ghandimathi (1975.b) and formation of plantlets from 1mm long apices, carried out by Carron et al (1989); the details of the latter were not given. They observed less contaminations of cultures, when smaller shoot tips were used.

Paranjothi and Ghandimathi (1975.b) used liquid M&S medium with casein hydrolysate and 3% sucrose, to culture apices in order to obtain plantlets. They were unable to grow apices on solid media. Since there was no multiplication, this report was only on rooting of shoot tips, which could easily be obtained in any media either solid or liquid, containing no exogenous cytokinins and/or in the presence of auxins.

The presence of exogenous hormones and their concentrations were effective on the number of axillary buds produced on shoot tips, but not on their elongation. Hormone free medium had the lowest percentage of axillary bud break, but the total of 3 ppm cytokinin in the medium was as good as 15 ppm. In fact, less cytokinin containing media favoured better maintenance of the shoot tips and good leaf growth.

Shoot tips harvested from both *in vitro* grown seed embryo derived plants and *in vivo* grown

rootstock derived materials, gave similar results. One difference between these two types of shoot tips, was the number of axillary buds on them; where seed embryo derived shoot tips contained only two axillary buds (Fig 5.a on page 38), while rootstock originated ones carried about ten or more buds on them (Plate 2.b on page 53).

However, the axillary bud proliferation was not successful with either type of shoot tips in the present work and therefore, the higher number of axillary buds on rootstock derived shoot tips, had no advantage over the other.

The advantage of using shoot tips instead of nodes for axillary shoot proliferation, was the higher number of axillary buds present in the shoot tips as compared to a node of the same length. Further, shoot tip contained the most active part of the shoot including the meristem.

Rahaman and Blake (1988a) also reported that the nodal explants were superior to shoot tips for shoot proliferation of seedling jackfruit (*Artocarpus hetorophyllus*). They compared the first and the second node but, found no significant difference.

Kito and Young (1981) compared shoot tips and nodes of sour orange (*Carrizo citrange*). Here, the nodes containing one axillary bud were obtained by removing 5mm shoot tips. But in this case, shoot tip explants proliferated significantly better than nodes, as reported for many other species. (Wu & Huang, 1990).

The unsuccessfulness of shoot tip culture, but satisfactory elongation of axillary buds of nodal explants, observed in the present studies of *Hevea* could be due to the strong apical dominance present in the apex. When an apex of about 10 mm size, was removed from a shoot tip, then the very first axillary bud of the resulting node started to grow suppressing the growth of axillary buds below that.

Since the nodal explants were found to be more suitable for axillary shoot proliferation, the rest of the present study was carried out with nodal explants.

In preliminary experiments, exogenous hormones were tested for axillary bud break and for their growth. Almost all the published work on node culture of *Hevea*, has been carried out by the research workers at Rubber Research Institute in Montpellier, France.

For the first time, in 1982, they reported the production of plantlets from nodes (Carron and Enjalric, 1982), and the procedure was named as 'microcutting'. A solid basic medium, the composition of which was not specified, but contained 5% activated charcoal and 6% sucrose, was used to culture nodes after soaking them in a solution of BAP and IBA (10 ppm and 5 ppm respectively) for two hours.

The bases of the resulting axillary shoots were dipped in a solution containing NAA and IBA (each at 5 ppm) for 5 days, to induce rooting and then cultured onto medium containing no growth substances. No proliferation was reported in this procedure, and each node produced a single plantlet.

Again in 1982, they found (Enjalric and Carron, 1982) that the presence of exogenous hormones (BAP-0.5, IBA-0.25 and GA_3 0.5 ppm) in the medium, had beneficial effect on elongation of axillary shoots, but only when the sucrose content of the medium was 2%. However, this difference was very small, when the media contained sucrose at 6%. The lengths of axillary shoots on both hormone containing and control media at 6% sucrose, had higher lengths of axillary shoots than those grown at 2% sucrose. This shows that the sucrose content in the medium is more effective than the presence of exogenous growth hormones for the elongation of axillary shoots. In this experiment MB medium was used as basic medium (Appendix 1).

In the present studies, the highest axillary shoot elongation was observed on hormone free medium at 2% sucrose. GA_3 was not used in any of the media. Therefore, Enjalric and Carron's observations must be due to the presence of GA3 in the medium, rather than the presence of auxins or cytokinins. Increasing lengths of primary axillary shoots with the increasing amount of sucrose in the medium, was observed in the present studies as well.

The effect of auxin was not tested in the present studies, but it was always present in the

medium unless otherwise stated. From the combinations of kinetin and BAP tested, it was found that the 2 ppm kinetin along with 1 ppm BAP, was optimum for axillary shoot growth with leafy shoots, although the maximum elongation was observed in control medium which contained no growth hormones.

The beneficial effects of using activated charcoal in the medium, on the shoot growth, reported by Enjalric and Carron(1982), was observed in the present experiments as well. Activated charcoal at 0.5% was as good as at 5.0% for the growth of leafy shoots and apical elongation.

It has been shown that activated charcoal has the ability to adsorb toxic metabolites released into the culture medium by the plant. Chevre et al (1983) used activated charcoal during the elongation and rooting stages of chestnut shoot tips, and found that it allowed better shoot elongation.

Preil and Engelhardt (1977) incorporated activated charcoal at the beginning of the culture initiation phase and observed prevention of multiple axillary shoot formation. This was a result of adsorption of BAP by the activated charcoal, which implies the requirement of higher levels of plant growth regulators in the presence of charcoal.

However, Carron and Enjalric (1982) did not use any hormones in the shoot producing medium, and therefore they could use activated charcoal as high as 50 g/l. When Enjalric and Carron (1982) used growth hormones in the medium, only 5 g/l activated charcoal was incorporated.

Carron and Enjalric did not report to have obtained root formation on hormone free medium as observed in the current experiments. The reasons for this may be the high content of charcoal in the medium and the pre-treatment of hormone solution which contained 10 ppm BAP and 5 ppm IBA.

Carron et al (1984) reported to have obtained more than one plant from a single node. This was true, but still, each axillary shoot was produced from a separate axillary bud, although they

were attached to the same node which was 3-4 cm long. Production of secondary axillary shoots from the nodes taken from primary axillary shoots, was also reported by them on a medium containing 5% charcoal and 6% sucrose. In 3-4 months, 50-70% plantlets were obtained in relation to the number of explants used, indicating that there had been no multiplication of shoots.

As shown by the results of the present work, exogenous hormones were not required at all for the axillary bud break and their elongation of primary nodes. But, if the quality of the shoots were concerned, the axillary shoots grown on the medium containing 2 ppm kinetin and 1 ppm BAP, were the best as stated earlier. The higher concentrations showed adverse effects on shoot elongation, survival rate and on leaf growth.

When the BAP alone was tested at 2,4,8 and 16 ppm, 2 ppm BAP gave the highest elongation, and the lengths of axillary shoots decreased with the increasing amount of BAP in the medium.

Carron et al (1989) used MB medium, as used by Enjalric and Carron (1982) for the culture of primary nodes after the hormone treatment. This contained 6% sucrose, but no charcoal this time. They claimed to have produced about 6 shoots in 5-6 subcultures by 'reinducing' the same mother explant on MB medium containing no growth hormones. But, by the end of this period the explant was dead. The only information available about the subculture period,was that the duration needed for the explant to produce primary axillary shoots, was 8 weeks. The other claim by Carron et al (1989) was successive production of axillary shoots using *in vitro* produced nodes. For the culture of nodes taken from the primary axillary shoots, they used Lepoivre medium (Quoirin and Lepoivre,1977) to which BAP and IBA were added, at 1 ppm and 0.01 ppm respectively. Efficiency of this method was given by monthly multiplication coefficiency which was 2-3.

This was the method used in the present studies with conventional hormones. But, as reported earlier, the growth of the axillary shoots produced in later passages was very poor compared to that of the primary axillary shoots, and also the proliferation rate obtained even on

the best medium found, was very low.

Koda and Okazawa (1980) also observed this situation with asparagus shoots, and the growth rate of the apices gradually reduced during the course of subculture. The shoots became thinner and the formation of lateral shoots was scarcely observed.

In the present studies, the cultures grown on hormone free media did not proliferate after 24 weeks of culture, showing the necessity of exogenous cytokinin for the axillary bud growth of *in vitro* harvested nodes. This was unnecessary for the growth of *in vivo* harvested nodes.

Although the experiments with conventional cytokinins gave some indication about hormonal requirements for the axillary shoot growth, the proliferation could not be continued beyond 44 weeks of culture, which includes the initial lag phase of about 12 weeks.

The MB medium described by Enjalric and Carron (1982), was used in early experiments with juvenile materials of *Hevea* by the author (Results are not reported here). But, no difference was observed between MB medium and 1/2 M&S medium for axillary bud break and the survival of the cultures, and therefore 1/2 M&S medium was chosen because of the convenience of use.

In the course of present experiments, woody plant medium (Lloyd and McCown, 1980) was found to be better than Murashige and Skoog medium.

In the first experiment, three levels of sucrose were also compared in addition to the basic media. Both media contained growth hormones which was found to be the best for axillary shoot growth of nodes (kinetin 2, BAP 1 and NAA 0.2 ppm).

From the two basic media tested, the survival rate of the explants grown on WPM medium, showed a significant difference from those grown on half M&S medium at all three levels of sucrose.

Several woody plants have been reported to grow better on lower salt concentrations than those described by Murashige and Skoog. Economou and Read (1984) reported sustained growth of shoot tip explants of azalea in a medium containing reduced levels of NH_4NO_3 and KNO_3.

M&S medium at 1/4 strength of major salts was the most satisfactory from the media tested

for the regeneration of red raspberry clones. (Pyott and Converse, 1981)

Perinet et al (1988) found that a low nitrogen medium (half of that of M&S) was better for the micropropagation of *Alnus incana* trees.

Preil and Engelhardt (1977) tried M&S media at full, 1/2 and 1/10 with 0.5, 1, 2 and 3% of sucrose. It was found that 1/10 M&S with 2% sucrose was the best for growth of azalea meristems. Further modifications of this basal medium showed that the increase of the organic compounds of the M&S medium up to full strength, may have a slightly positive effect on some varieties.

Many woody species are known to be sensitive to salt (NaCl), and for this reason WPM was designed to minimize chloride levels by using sulfate salts where possible.

McCown and Sellmer (1987) suggested that the response of plant material to the basic medium, was highly genotype dependant. One genotype of poplar could be maintained indefinitely on WPM medium, but the growth was poor. When these were transferred onto M&S medium, immediate improvement of biomass production was observed. Since one major difference between M&S and WPM is the level of macroelements, WPM medium was supplied with NH_4NO_3 to the level of M&S. The response to this medium was similar to that of M&S.

Read et al (1982) reported that WPM was superior to M&S for the culture of *Salix* and *Alnus*. WPM medium was successfully used by Heiman and Preece (1983) in shoot tip culture of *Fraxinus*.

Garton et al (1981) achieved rapid clonal propagation of *Alnus glutinosa* using juvenile lateral buds on WPM with 0.22 ppm BAP. Rugini and Fontanazza (1981) found that 1/2 strength M&S medium was better than full strength for shoot proliferation of 'Doke agogia' olive. At full strength, proliferation was reduced and shoot growth stunted. Cheng (1988) used twofold and fourfold diluted basal medium when the growth was retarded in the normal strength of the medium. Barnes (1985) obtained highest multiplication of *Rhododendron* nodes on WPM medium. Anderson (1975) reported the necessity of reduced potassium concentration in the M&S

medium, for sustained shoot growth of *Rhododendron* seedlings and shoots. The KNO_3 concentration was reduced to 950 ppm and the NH_4NO_3 increased to 2000 ppm.

On the other hand, Fink et al (1986) reported that the shoot tips of 'Pioneer' elm cultured on M&S medium, maintained bright green colour, and some shoot tips produced multiple shoots. Most of the original shoot tips failed to survive on WPM medium regardless of the cytokinin concentration.

Compared to the MB medium used by Carron and Enjalric (1982), WPM contains much less salts as macroelements. NH_4NO_3 present in WPM is less than half of that in MB medium while KNO_3 and NaH_2PO_4 H_20 are totally absent. The amount of $CaCl_2$ in the WPM is about $1/_{10}$ of that of MB medium (Appendices 1 & 2). No further experiments were carried out in the present work with media components, but adenine sulfate dihydrate was used in all media at 100 ppm. Anderson (1975) incorporated adenine sulfate into culture media at 80 ppm for axillary bud break and sustained shoot growth. Cohen and Cooper (1982) too used adenine sulfate at 40 ppm, and they found a dramatic improvement in shoot growth.

The most common carbon sources being used in the culture media are sucrose and glucose. Other carbohydrate sources have been tested in numerous experiments, but they rarely prove to offer any advantage over the two main sugars. In many cases, it is unnecessary to consider anything other than sucrose for this purpose, at a concentration of two or three percent (20-30 g/l). This has been proved by the fact that most of the tissue culture media contained sucrose within this range. But there are exceptions too. Tian (1984) used only 1% sucrose in the medium for *Hibiscus rosa sinensis*. For the axillary shoot growth of crape myrtle 1.5% sucrose was sufficient (Huang, 1984b). *Ginkgo biloba* nodes were grown with 4% sucrose (Luo, 1985), and for the axillary bud growth of *Leucaena leucocephala*, 5% sucrose was used in the medium (Huang, 1984 a). Carron and Enjalric (1982) used 6% sucrose for the *Hevea* node cultures. For birch micropropagation sucrose was used at, as high as 10% (McCown and Amos, 1979).

From the three levels of sucrose tested in the first experiment of the present work, the effect of sucrose on the elongation of axillary shoots was very clear. The lengths increased with the increasing level of sucrose in the medium from 2% to 8%, in both M&S and WPM media. Enjalric and Carron (1982) have also reported that 6% sucrose was better than 2%. The effect of sucrose was not clear in later passages, but the shoot growth at both 4 and 8% was better than that of 2%. Sice the difference between 4 and 8% was not large, 4% was used in later experiments. The level of sucrose was tested again with clonal materials (as appeared in Chapter 5), where 0, 2, 4, 6, 8 and 10% sucrose were tested. It was found that 6% sucrose was slightly better than 4%. Sucrose at 8% also showed good shoot growth and leaf growth, but 6% was chosen to use, because on one hand it was as good as 8% and on the other hand the leaves grown at 10% sucrose were vitrified.

Even though WPM medium was found to be better than M&S medium for the axillary shoot growth and for the survival rate of the shoots, the propagation rate was still very low with conventional growth hormones. Therefore, thidiazuron was tested with nodal explants since it had worked well for other species.

The compound thidiazuron, N-phenyl-N'-1,2,3-thidiazol -5-ylurea (or commonly known as Dropp), is known to be a plant growth regulator and has been used as a cotton defoliant at experimental scale. However, the biological preparation of this compound had not been reported, including cytokinin activity, until the report of Mok et al(1982). They compared the relative activity of eight cytokinin-active adenine derivatives, in promoting the growth of callus tissues of *Phaseolus lunatus* and showed that thidiazuron was a highly active cytokinin.

The mode of action of thidiazuron is not yet clear; it may act directly at the site of adenine-type cytokinin action or, it may influence endogenous cytokinin biosynthesis (Thomas and Katterman, 1986). The chemical structure of thidiazuron is as in Figure 26.

Kerns and Meyer (1986) observed initiation of shoot proliferation when the shoot tips of

Acer x freemanii were transferred to a medium with 0.0022 - 0.011 ppm thidiazuron.

The strong apical dominance and apparent suppression of axillary meristems, which prevented proliferation, was observed by them over four years, while numerous treatments were tested including kinetin, BAP, 2iP, IBA, TIBA, GA_3 etc. The growth of shoot tips continued as single shoots and did not proliferate, as reported by Welsh and Sink (1982) for red maple.

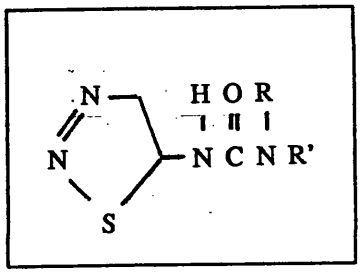


Fig 26. The chemical structure of thidiazuron (Mok et al,1982).

The range of thidiazuron tested for *Acer x freemanii*, was from 0.001 to 1.125 ppm and the optimum range for shoot proliferation was 0.0022 to 0.011 ppm. The effect of BAP was also tested by incorporating BAP at 0.225 ppm into the medium together with thidiazuron. It was found that the interaction of BAP enhanced the effect of thidiazuron, though it was not statistically significant. Using 0.002 ppm thidiazuron and 0.225 BAP in the medium, up to 10 shoots from each explant (shoot tips or basal nodes) could be separated and subcultured. Continuous proliferation was obtained in this method, but no information about subculture period or proliferation rate was reported.

In one experiment carried out in the present studies with thidiazuron, 0.1 and 0.02 ppm thidiazuron were compared with S-1 hormones (kinetin 2, BAP 1 and NAA 0.2 ppm). BAP was incorporated at 0.2 ppm and NAA was present at 0.2 ppm together with thidiazuron. Results of

this experiment showed that thidiazuron stimulated multiple axillary shoots on nodal explants, while conventional hormones produced only single axillary shoots. This finding of the effect of thidiazuron was very promising for the axillary shoot proliferation of *Hevea* and obviously encouraging compared to the results obtained with conventional hormones for years.

To optimize the level, a range of thidiazuron 0, 0.002, 0.02 and 0.2 ppm were tested. This time BAP was not incorporated, but NAA was present at 0.2 ppm. Concentrations beyond 0.2 were not tested because when comparing 0.1 and 0.002 ppm, the higher concentration showed poor shoot quality and leaf growth. On the other hand, the other workers who worked with thidiazuron, also used very low concentrations. Seed embryo nodal explants grown on 0.001 ppm thidiazuron containing medium, behaved as they were on control medium, and produced only single axillary shoots. Therefore, concentrations below 0.002 ppm were also not tested.

From this range tested, 0.02 ppm showed the maximum proliferation. The shoot doubling times for the media containing 0.002, 0.02 and 0.2 ppm thidiazuron were 39, 23 and 28 days respectively.

45% of the shoots grown on control medium and 25% of them on 0.002 ppm thidiazuron produced roots. Although 0.002 ppm thidiazuron was not strong enough to stop root formation on shoots, it was high enough to induce multiple axillary shoots. Root formation was not observed at this concentration when the BAP at 0.2 ppm was present.

Very condensed clusters of buds were produced on 0.2 ppm thidiazuron. But, these clusters started to produce normal multiple axillary shoots after 8 weeks of their transfer onto 0.002 ppm thidiazuron medium.

The multiple axillary shoots produced on thidiazuron media, developed roots on their transfer onto control medium. The time required for the disappearance of thidiazuron activity on the transfer onto control medium, was dependent on the concentration of thidiazuron in the medium where they had been grown. Also the cultures grown on control medium as single shoots, started to produce multiple axillary shoots with their transfer onto thidiazuron containing

medium.

The present finding of the activity of thidiazuron is in agreement with the findings of Nieuwkerk et al (1986). They observed better shoot proliferation of apple in the presence of thidiazuron. They tested 0.00022, 0.0022, 0.022 0.22 and 2.2 ppm thidiazuron and the control medium contained 1 ppm BAP.

Thidiazuron at 0.022 and higher concentrations caused thickening of the stems in about 6 weeks of culture. This was true for both shoot tips and nodes. By the end of ninth week of culture, the shoots produced on 2.2 and 0.2 ppm concentrations were too small and numerous to count. The mean number of shoots produced at 0.022, 0.0022 and 0.00022, were 4.2, 1.8 and 1.7 respectively. For the control medium it was 3.5. Also, the shoots formed in thidiazuron containing media, were tended to be shorter than those produced in the presence of BAP alone.

In the three higher concentrations of thidiazuron, large clumps of shoots formed, some apparently growing adventitiously out of a firm callus, that was difficult to to be distinguished from the enlarged stem tissue at the center of the clump.

A similar situation was observed with *Hevea* too in the present work. When the large clusters of axillary shoots were separated into smaller ones, new axillary shoots always emerged from the bases of the clusters, the origin of which were difficult to determine (Plate 11).

As reported by Nieuwkerk et al(1986), after transferring cultures from thidiazuron containing medium to cytokinin free or growth regulator free medium, proliferation ceased within 3 weeks for most treatments, but continued for up to 15 weeks for explants that had been on 0.22 or 2.2 ppm thidiazuron containing media.

Kerns and Mayer (1985) also reported the effects of thidiazuron on shoot proliferation of red-silver hybrid maples. The prolifics obtained at 0.022 - 0.11 ppm thidiazuron could be separated into 5-7 new propagules at 6 weeks intervals.

Thidiazuron was found to be effective on callus induction and organogenesis as well. Capelle et al (1983) reported that thidiazuron was extremely active in stimulating callus growth of

Plate 11. Multiple axillary shoots produced at 0.02 ppm thidiazuron. Results after 20 weeks of culture.

Phaseolus lunatus.

Fiola et al (1990) found that thidiazuron was significantly more effective than BAP on inducing organogenesis from detached *Rubus* cotyledons and leaves. The optimum concentration of thidiazuron for organogenesis from cotyledons was 1.1 to 2.2 ppm and for leaves 1.1 to 4.4 ppm. These were much higher than the ranges found optimum for shoot multiplication.

The effect of thidiazuron with most of the plants showed, that it could be used to proliferate shoot tips or nodes *in vitro*, and that thidiazuron was effective at much lower concentrations than conventional cytokinins like BAP. From the cytokinins tested in the present studies thidiazuron was the most effective, where the continuous shoot proliferation was observed.

Apart from the main difference observed between the shoot tips and the nodes for axillary shoot production, the growth stage of the shoot from which the explants were taken and the position of the node in the shoot, also had effects on the production of axillary shoots.

Three growth stages were identified according to the growth pattern of *Hevea* which always occurs in flushes. They were very young actively growing phase, intermediate phase and the stationary phase (Figure 18 on page 76 and Fig.50 on page 180). Maximum elongation of primary axillary shoots were obtained from the nodes harvested from the shoots which were in the stationary phase of growth. The media used contained no exogenous growth hormones, and therefore, the axillary bud break and their elongation were totally dependent on the amount of endogenous hormones carried with them.

Stationary state of growth, which lasts about five weeks, is the phase just before the growth of a new flush. The physiological changes that occur inside the tissues during this period, must be towards the production of new shoot growth. Therefore, it is reasonable for the explants taken from this growth stage, to show the best results in producing axillary shoots.

The position of the node in the shoot also affected the production of axillary shoots. In the experiment carried out with juvenile materials of *Hevea*, the nodes were numbered from 0 to 7 according to their position on the shoot, 0 being the shoot tip. The sizes of the nodes varied because the internodal lengths were not uniform. Growth hormones were not used in the media for the growth of primary axillary shoots, but for the induction of secondary axillary shoots from the nodes harvested *in vitro*, the presence of exogenous hormones was necessary.

The lengths of axillary shoots of primary nodes increased down along the shoot (where the nodes were collected) some far, and then started to decrease.

The increase of the axillary shoot lengths with the increasing distance from the shoot apex, could be due to the gradual decrease of the extent of apical dominance which is present in the apex. The decrease of axillary shoot length after the maximum length, could partially be due to the physiological state of the axillary buds, inactivity or dormancy gained with their age.

The effect of the position was tested with mature materials of *Hevea* too, and it was found that the nodes without a leaf attached to it, were 'dormant' or could not be induced to produce axillary shoots *in vitro*.

The main shortcoming with conventional hormones in the axillary shoot proliferation, was the poor axillary shoot growth and elongation of nodes collected *in vitro*, after secondary or tertiary nodes. The effect of GA_3 was tested on the elongation of these secondary and tertiary axillary buds produced *in vitro* using conventional hormones. Nodes were grown on hormone free media prior to testing the effect of GA_3. Initially 0, 0.5, 1, 2, 4 and 8 ppm were tested and later one more level, 16 ppm was added. The effect of GA_3 was tested together with S-1 hormones as well (kinetin 2, BAP 1 and NAA .2 ppm).

The effect of GA_3, if there was any, could not be observed in the present work. About 40% of the cultures grown on 16 ppm GA_3 medium, showed some elongation, but no continuous growth of axillary shoots was observed.

Enjalric and Carron (1982) found some elongation of axillary shoots in the presence of GA_3. The media contained BAP 0.5 ppm, IBA 0.25 ppm and GA_3 0.5 ppm. But, this elongation was observed only for the growth of primary axillary shoots at 6% sucrose, and therefore this results could not be compared with the present work which was carried out with secondary and tertiary nodes. In the present work, maximum elongation of primary axillary shoots was obtained in the absence of growth hormones, but the effect of GA_3 on the elongation of primary axillary shoots was not tested in the present work.

Cresswell et al (1982) reported that the concentration of GA_3 from 1 ppm to 10 ppm, lead to a considerable elongation of the internodes of the shoots. The internodal length increased from 1 mm to 15 mm in less than 15 days. But, after this the apex of the shoot died and the leaves became fragile and fell at the slightest shock. However, media containing 0.5 ppm GA_3, though causing less elongation, produced healthier shoots and better roots.

The effect of rooting on the elongation of axillary buds of secondary and tertiary nodes were also tested in the course of current experiments. It was observed that there was a significant effect of rooting on the elongation of axillary shoots of the resulting nodes. The primary nodes harvested *in vivo* showed no difficulty in axillary shoot elongation. The nodes taken from rooted plants, although originated *in vitro*, seems to behave in the same way. But, the disadvantage of this is that the rooting passage too, requires at least 8 weeks and therefore, shoot multiplication rate can be very low.

Rooting of *in vitro* produced shoots of *Hevea*, was rather easy compared to the other stages in the micropropagation procedure. The shoots produced roots even on the control medium with no growth hormones. Carron and Enjalric (1982, 1984 and 1989) obtained rooting by dipping the bases of shoots in a solution of IBA and NAA (each at 5 ppm) for 5 days, followed by culturing them onto hormone free solid medium containing 6% sucrose. They also used commercial rooting powders which contained NAA, for rooting of juvenile *Hevea* shoots.

Manzanera and Pardos (1990) used hormone free low salt media with 4% sucrose for rooting of cork oak shoot tips. Low concentrations of IBA added to the medium , gave the best results.

In the present studies, 6% sucrose was found to be the optimum for shoot growth. For rooting, 4% sucrose was used with half M&S salts and 2 ppm IBA. Since this combination gave satisfactory results, no further experiments were carried out on rooting. The roots obtained were as strong as those of embryo cultured plants and contained lateral roots . Although the best root formation was observed in absence of charcoal, the shoot growth was always better in the presence of charcoal. IBA was found to produce good roots on *Pistacia* shoots (Barghchi and Alderson, 1983), and this was supported by the observation of Barazi and Schwabe (1982) on rooting of softwood shoots of *Pistacia.*

Engelmann et al (1987) also reported the beneficial effects of charcoal (2 g/l) for shoot maintenance. They could induce roots on *Cunninghamia lanceolata* shoots on a medium containing 3 ppm IBA without any difference between mature and juvenile genotypes.

Weaning of rooted plantlets of *Hevea*, was not difficult provided that high humidity was maintained for the first two weeks. Since the roots were produced on solid media, agar should be washed off well, otherwise it may promote fungal contaminations around the plant under high humid conditions.

In conclusion, a protocol can be suggested for continuous micropropagation of *Hevea.*

1. Explants - Nodes removed from the actively growing part of the shoot.
2. Culture medium - Solid WPM medium supplied with 6% sucrose and thidiazuron (in the range 0.02 - 0.002 ppm).
3. Subculture period - 4 weeks
4. Culture conditions - At 25+2 $^{\circ}$C under 16 hour photoperiod.

Chapter.4
Axillary Shoot Production Potential of Mature Materials of *Hevea*

4.1. Results

The work reported in this chapter was carried out with mature, clonal materials of *Hevea* belonging to five clones.

Preliminary problems such as difficulty in sterilizing plant materials and culture establishment, are discussed in detail. Variables such as explant type, basic medium and sucrose level of the culture medium were also tested.

4.1.1. Disinfection, Phenolic Browning and Culture Establishment of Shoot Explants.

Shoot tips and nodes of mature origin were more difficult to sterilize and establish in culture compared to juvenile materials of *Hevea*. Because on one hand, they seem to carry a microbial flora living on the surfaces of the shoots and in the tissues, and on the other hand, they contained a very high amount of phenolic compounds, and therefore they were very sensitive to sterilants.

(a). Sterilization with NaOCl

At first, the sterilization of explants was carried out with NaOCl. All possible combinations of 5%, 10%, 15% and 20% of NaOCl with 5, 10, 15 and 20 minutes durations, were tested. NaOCl solutions were made with sterile distilled water under the Laminar flow cabinet, and 0.1% Tween 80 was added as the surfactant. All the glassware used to make solutions, were sterilized in the autoclave prior to use.

Explants used were collected from all five clones. Solid M&S Medium, at half strength, supplied with 2% sucrose and without growth hormones, was used. Media were made as slants in glass tubes, in order to obtain maximum surface area, and the shoot tips were placed horizontally on the medium. There were 15 replicates for each sterilization treatment. Results could be seen within the first week of culture. The percentage contamination rate and phenolic browning of cultures are given in Table 6.

		Concentration of NaOCl.			
Time mins		5%	10%	15%	20%
5	Contaminations Phenolic browning	100 50	90 60	70 90	50 100
10	Contaminations Phenolic browning	100 65	75 60	50 90	25 100
15	Contaminations Phenolic browning	100 75	50 80	55 100	25 100
20	Contaminations Phenolic browning	100 75	50 100	50 100	25 100

Table 6. Percentage of contamination rate and phenolic browning of shoot tip explants, sterilized with NaOCl (n=15).

As shown in Table 6, both contaminations and phenolic browning were observed in each treatment. 5% NaOCl was totally ineffective to sterilize shoot tips, but phenolic browning was still observed. The higher concentrations of NaOCl sterilized about 75% of the explants, but at the same time they killed explants by causing phenolic browning which was observed at 100%. All these treatments were repeated at least once or twice, individually or in groups, but the same results were obtained always. 10% NaOCl for 10 minutes and 15 minutes were the only acceptable treatments which gave 25% and 50% of clean cultures respectively, although phenolic browning too was observed at about 60% and 80% .

The treatment with 10% NaOCl for 10 minutes was tested again, but this time they were washed in 70% EtOH for 2 minutes prior to sterilize in 10% NaOCl. About 50% of the cultures were free of contaminants, but the browning remained as high as 60%.

(b). Phenolic Browning.

Sterilization method could not be improved because of the high level of phenolic browning observed which caused eventual death of explants. Therefore, the following treatments were tested with shoot tip explants in order to control phenolic browning.

(i). The strength of the basic medium (full, 1/2 and 1/4 M&S).

(ii). Incubation of cultures at low temperature under the dark.

(iii). Use of antioxidents.

(iv). Use of activated charcoal.

(v). Frequent transfer of cultures onto fresh media.

(vi). Use of PVP.

(i). The Effect of the Strength of Basic Medium on Browning.

Shoot tip explants were sterilized with 70% EtOH for 2 minutes, followed by 10% NaOCl for 10 minutes. M&S media was prepared in Petri dishes, at 1/4, 1/2 and full strength with 2% sucrose and no growth hormones. There were 20 replicates.

After 2-3 days of culture, all the shoot tips cultured onto full M&S medium turned brown. The colour intensity of the medium surrounding the explant too, was high compared to the other two media. Browning was observed on the other two media as well, but to a lesser extent, and there was no difference between 1/4 strength and 1/2 strength on browning. Since there was no advantage in using 1/4 strength, 1/2 M&S was chosen for the rest of the experiments.

(ii). Incubation of Cultures at Low Temperatures under the Dark.

Shoot tips, 4-5cm long, were sterilized with 70% EtOH for 2 minutes followed by 10% NaOCl for 10 minutes, as before. Half strength M&S solid medium supplied with 2% sucrose, was used with no growth hormones. Cultures were incubated at 20°C in the dark, while control cultures were kept at 25±2°C under 12 hour photoperiod. There were 20 replicates.

Results after one week, showed that the incubation at low temperature has some effect to reduce the rate of production of phenolic browning. Control cultures showed browning at 100%, while cultures incubated in the dark showed about 40% of browning. The rest of the 60% also showed browning of the medium, but the stems were still green. Contaminations were observed in about half of the cultures.

At the end of the first week, all uncontaminated cultures, from the control and treated, were transferred onto fresh media. Half of the cultures, that had been incubated in the dark at low temperature, was continued to incubate under the same conditions, while the other half was returned to normal temperature, 25±2°C. After the second week of culture, the difference observed between the three sets of cultures was very little. Browning was present at 100%, and at the end of the third week the whole lot had to be thrown away.

(iii). Use of Antioxidents.

A mixture of ascorbic acid and citric acid, 100 and 150 ppm respectively, was used as antioxidents in this experiment.

Antioxidents as a Pretreatment.

In this experiment shoot tips were sterilized as explained in the previous experiment (i), and then soaked in the antioxident solution. Two time periods were used, 1/2 hour and 1 hour. Solid M&S medium, at half strength, supplied with 2% sucrose and no growth hormones, was used in tubes, made as slants. The cultures were incubated in the light at 25±2°C. Control was shoot tips cultured with no antioxident treatment, and there were 20 replicates.

Contrary to expectations, more phenolic exudates was observed in treated cultures. There was no difference between the two soaking times, 1/2 hour and 1 hour, for the extent of browning. At the end of the first week, almost all the cultures turned brown and eventually died. Contaminations were observed in about 50% of the cultures.

Incorporation of Antioxidents into the Culture Medium.

The same mixture of antioxidents was used in this experiment. Both solid and liquid media were made in tubes with antioxidents. Half M&S solid medium with no antioxidents, was used as the control. 25 explants were cultured into each of the 3 media. In liquid medium, cultures were supported on folded filter papers vertically. In solid media, they were placed horizontally on the agar as before. Explant sterilization and the culture procedure were same as explained before.

After 4-5 days of culture, browning was observed in all 3 media. Cultures on control medium and liquid medium with antioxidents, showed no difference at all in the extent browning. Those on solid medium with antioxidents, showed some beneficial effect of antioxidents in reducing browning. About half of the cultures on this medium had their green colour, although the surrounding media were stained brown. They were transferred onto same fresh media at the end of the first week. More phenolic exudates was observed during the second week, and only about 10% of the cultures on solid antioxident containing medium survived at the end of the second week.

(iv). Use of Activated Charcoal.

In this experiment, activated charcoal was used at 0.5% and 1%. Again, the sterilization of shoot tips, the basic medium composition and the culture procedure were as before. Replication was 15. Media were prepared as follows: both solid and liquid media free of charcoal were the control.

% of Charcoal

Medium state	0%	0.5%	5.0%
Solid			
Liquid			

After 1 week of culture, most of the cultures grown on solid medium containing charcoal,

remained green. Liquid medium with charcoal, had no visible effect in reducing browning. Cultures grown on this medium, showed browning as much as those grown on control media. Therefore, the shoot tips grown only on solid charcoal medium were transferred onto fresh media. But, the effect of charcoal was reduced with time, and at the end of the third week only two cultures survived. Activated charcoal was effective to slow down the process of browning of shoot tips, but it was not effective to prevent them from dying. The high contamination rate always prevented proper judgement on the variable tested.

(v). Frequent Transfer of Cultures onto Fresh Media.

In this experiment, the sterilization of shoot tips and the culture conditions were same as in the previous experiments. Half strength solid M&S medium supplied with 2% sucrose, was used, and the media were made in 9 cm Petri dishes instead of tubes. There were 15 replicates. During the first day of culture, shoot tips were transferred on to fresh media about five times, at the 1st, 3rd, 6th, 10th, and 15th hour of culture. From the second day, they were transferred daily up to a week and then weekly up to 4 weeks. Control cultures were left without transfer until the end of the first week and then transferred weekly.

During the first day, brown exudates were observed in the media at each time of transfer. But the extent of browning was reduced with the number of transfers. However, browning was observed throughout the first week.

All the cultures left without transfer were dead at the end of the first week. More than 50% of the cultures which were transferred frequently, survived the 4^{th} week of culture. But the contamination rate remained as high as 50%, and therefore only about 25% of shoot tips remained free of contaminations and browning.

Frequent transfer of cultures onto fresh media was the most effective to reduce browning of shoot tips, from the treatment tried so far.

(vi). Use of Polyvinyl Polypyrrolidone (PVP).

Two types of PVP were used in the experiments here; insoluble PVP and water soluble PVP.

Insoluble PVP.

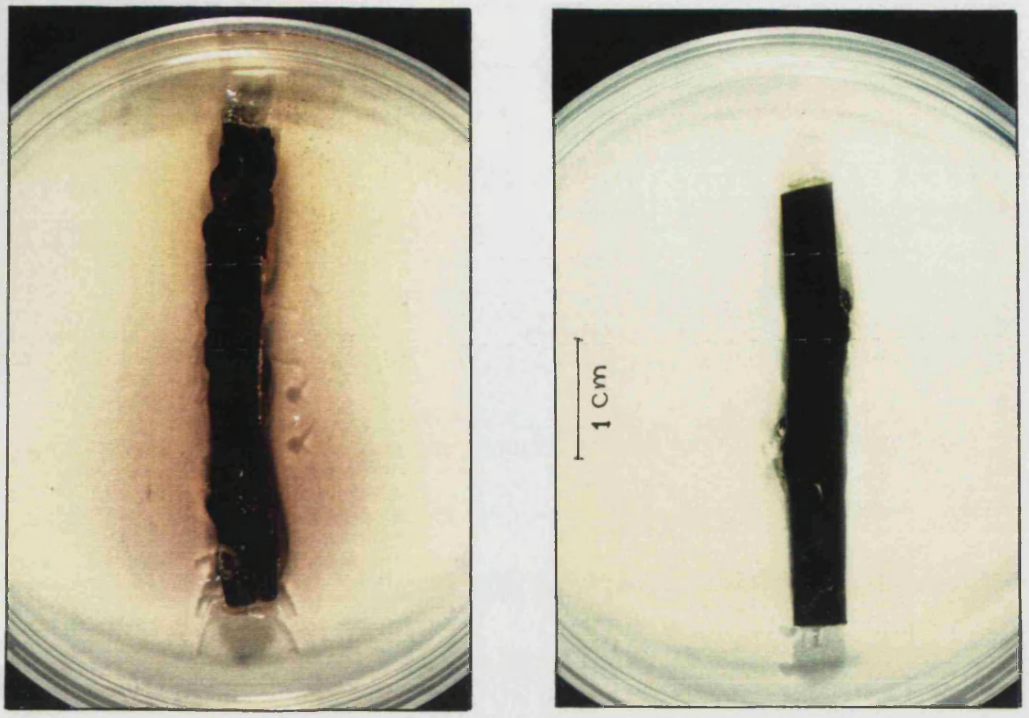
In this experiment, PVP was used as a solution of 100 ppm, to soak explants before culturing, and also incorporated into the culture medium at the same concentration, in a separate experiment. Both solid and liquid media were tried here. When explants were soaked in PVP, they were cultured onto solid media containing no PVP. Explant sterilization, culture conditions and the medium compositions, were the same as in the previous experiments. Solid media were made in Petri dishes and liquid media were made in tubes. There were 15 replicates.

No beneficial effect was observed either by soaking or by incorporating insoluble PVP into the growth medium to reduce browning of shoot tips. In fact, both soaking in PVP solution and use of liquid medium had adverse effects.

Water Soluble PVP.

This experiment was carried out exactly in the same way as before, but water soluble PVP was used. The results obtained with soaking treatment were similar to those observed with insoluble PVP. Cultures on PVP containing Liquid medium too turned browned as those on the control medium. But, the cultures grown on solid medium containing PVP, showed a remarkable improvement. About 75% of the cultures on this medium remained green, although the surrounding agar medium was stained light brown. After 1 week, the cultures were transferred to fresh media of the same composition.

Phenolic browning was effectively controlled by incorporating PVP at 100 ppm into the medium, but at the end of the third week only about 30% of the cultures survived, because more than half of the cultures were contaminated.



a. b.

Plate 12. The effect of PVP,(a) without PVP in the medium and (b) with PVP in the medium, at 100 ppm. Results after 24 hours.

Optimizing the Level of PVP in the Medium Using Water Soluble PVP.

In the previous experiment, only one concentration of PVP was tested which controlled browning successfully. In this experiment, one more level of PVP was tested, 200 ppm, and the control medium contained no PVP. Only solid media in Petri dishes were used, since liquid medium was found to be not effective. Sterilization, culture conditions and the rest of the medium composition were used as before.

Cultures grown on PVP free medium showed severe browning, while those on both 100 ppm and 200 ppm PVP containing media showed no browning in about 75% of the cultures, and no detectable difference was observed between the two levels of PVP. Therefore, 100 ppm water soluble PVP was used with solid medium in later experiments.

(c). Sterilization with $HgCl_2$.

In all the experiments reported so far, the explants were sterilized in 70% EtOH for 2 minutes followed by 10 minutes in 10% NaOCl. With this sterilization procedure, the maximum number of clean cultures obtained was about 50%.

In this experiment, a solution of $HgCl_2$ was used as the sterilant. At first, the following 4 treatments were tested. Tween 80 was incorporated at 0.1%. Sterilized distilled water was used to make solutions.

Concentration of $HgCl_2$

Time	Treatment number	0.1%	0.2%
	5 minutes	1	2
	10 minutes	3	4

Solid M&S medium at half strength supplied with 2% sucrose and 100 ppm water soluble PVP, was used in Petri dishes. There were 15 replicates.

When culturing shoot tips, two 1 mm thick slices removed from the base of the shoot tip, were indexed onto NAA and PDA plates for easy detecting of contaminations.

Results after the first week of culture are given in Fig 27 (see also Appendix 15).

As shown in Fig 27, the use of $HgCl_2$ as a sterilant not only increased the number of clean cultures, but also reduced the amount of phenolic browning. From the 4 treatments tested, 0.2% $HgCl_2$ for 10 minutes gave the best results; 80% of clean cultures could be obtained and phenolic browning was only 10%. Using 70% EtOH for 2 minutes as a pre-wash, reduced the contamination rate further. In most of the instances, 100% clean cultures were obtained.

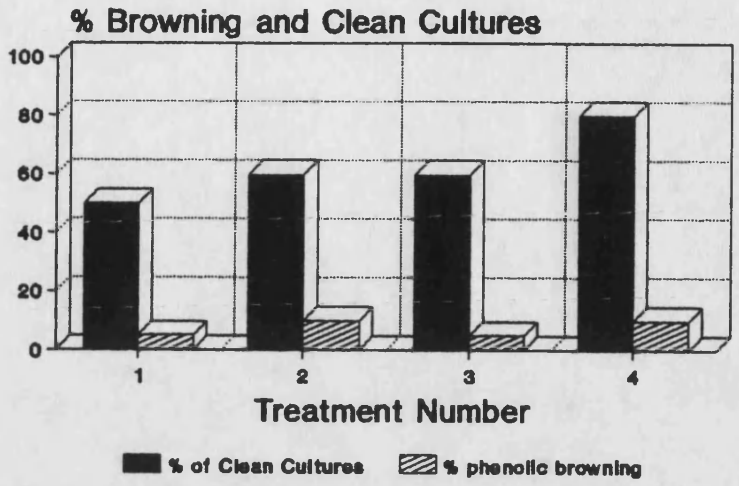


Fig 27. Percentage of clean cultures and phenolic browning of shoot tip explants sterilized with $HgCl_2$ (n = 15).

(d). The Effect of Explant Type, Physiological State and the Clone on Phenolic Browning.

(i). The Effect of the Explant Type on Phenolic Browning.

Shoot tips and nodes harvested from a mixture of clones were compared in this experiment. 4-5 cm long shoot tips and node were sterilized with 70% EtOH for 2 minutes followed by 0.2% $HgCl_2$ for 10 minutes. Half strength solid M&S medium supplied with 2% sucrose, was used in Petri dishes. PVP was not incorporated into the media. There were 15 replicates from each type of explants.

Results after 1 week, showed that the amount of phenolic browning observed with nodal explants, was much less than that observed with shoot tip explants. Phenolic browning was present in most of the nodes and in all of the shoot tips. In nodes, the explants remained green, although the surrounding medium was stained. But with shoot tips, the explants too turned brown together with the medium. In about 90% of the shoot tips, browning was lethal.

The browning of nodes could be successfully successfully by incorporating PVP into the medium, as explained before. But with shoot tips phenolic browning which was not harmful, was observed even in the presence of PVP.

(ii). The Effect of the Physiological State of the Explant on Phenolic Browning.

Nodal explants at 'young' and 'mature' state of growth, were used from clone RRIC 100. Nodes at their 'young' state of growth, had very tender stems and very young unexpanded leaves. At 'mature' state, the stems were woody and contained mature expanded leaves.

Explants were sterilized with 70% EtOH and 0.2% $HgCl_2$ as before. The same medium used in the previous experiment, was used and there were 10 replicates.

Results after 1 week showed that the phenolic browning of 'young' nodes was always higher than that of the 'mature' nodes. With 'young' nodes, browning was observed at 100%, while with 'mature' nodes it was about 50%.

PVP in the growth medium at 100 ppm, prevented browning of 'mature' nodes totally, but 'young' nodes still produced phenolics in about 25% of the cultures, although this was not as severe as with shoot tip explants.

(iii). The Effect of the Clone on Phenolic Browning.

In this experiment, clones PB 86, RRIC 100, RRIC 110, RRIC 117 and RRIC 121 were used. Both shoot tips and nodes were cultured, and the rest of the experiment was as in the previous experiment.

As observed before, more phenolic browning was again observed with shoot tips, and therefore the difference between clones was difficult to detect. However, the sequence of clones according to the extent of browning was as follows:

RRIC 110 > RRIC 121 > RRIC 117 = RRIC 100 > PB 86

According to the expectations, with nodal explants, the extent of browning was much less

than that observed with shoot tips. However, the sequence of clones in descending order of the amount of phenolics produced, was similar to that obtained with shoot tips.

4.1.2. Choice of Explants.

The experiments on choice of explants were carried out in three stages:

(a). Comparison of Shoot Tips and Nodes for Axillary Shoot Growth.

The axillary shoot production potential of shoot tips and nodes, was compared with juvenile materials as discussed in the Chapter 3. The same experiment was carried out with shoot tips and nodes of clone RRIC 110.

Half strength M&S medium supplied with 2% sucrose, 100 ppm PVP and S-2 hormones (kinetin 7.5, BAP 3.75 and NAA 0.2 ppm), was used in Petri dishes. Explants were sterilized with 70% EtOH for 2 minutes, followed by 0.2% $HgCl_2$ for 10 minutes. Cultures were incubated in the light at 12 hour photoperiod. There were 12 replicates.

According to the expectations, the results observed were similar to those obtained in the experiment with juvenile materials. After 4-6 weeks of culture, axillary bud break occurred in both shoot tips and nodal explants, but the elongation of them was observed only with nodes (Plate 13).

The axillary buds on shoot tips did not elongate more than 2-3 mm, whereas 25-30 mm elongation was observed in nodes. Normal leaf growth was seen with axillary shoots of nodal explants, but not of shoot tips. Apical elongation of about 5-10 mm occurred in shoot tip explants. The results of this experiment confirmed those of the experiment with juvenile materials, indicating that the productive explants for axillary shoot proliferation was nodal explants. As can be seen from Plate 13 a, from the three axillary shoots on the node, the highest elongation had occurred from the axil located farthest from the apex.

Plate 13. Comparison of **(a)** shoot tips and **(b)** nodes for axillary shoot growth. Results after 20 weeks.

(b). The Effect of the Type of Node.

Generally, two types of nodes could be identified in a shoot as shown in Figure 28. For the ease of identification they were called as 'active' nodes and 'dormant' nodes, during the experimental period.

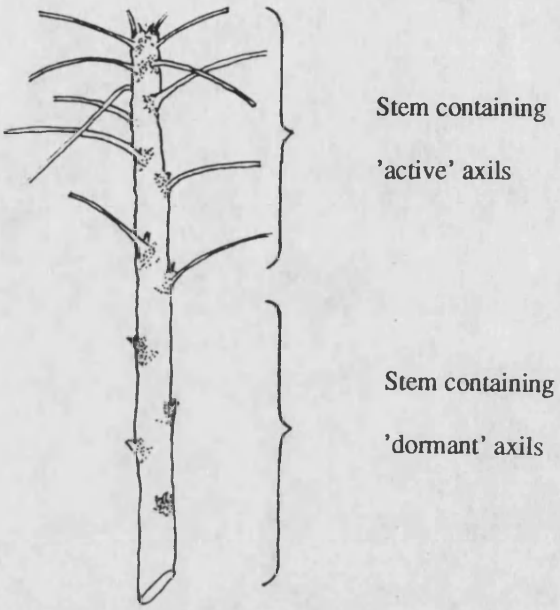


Figure 28. The two types of nodes in a shoot as can be identified by their external appearance

The main difference observed between the two types of nodes, was that 'active' nodes contained a leaf attached to them, and 'dormant' nodes were without a leaf. In this experiment, these two types of nodes were compared for their ability to produce axillary shoots.

Four media were used, S-0, S-1, S-2 and S-3, compositions of which are explained in Chapter 3 on page 49. The basic medium was half strength M&S supplied with 2% sucrose and 100 ppm PVP. Media were prepared in Petri dishes, and there were 10 replicates. Sterilization and culture procedure were as in the previous experiment.

Results obtained at 16 weeks of culture, are shown in Fig 29 a & b (see also Appendix 16).

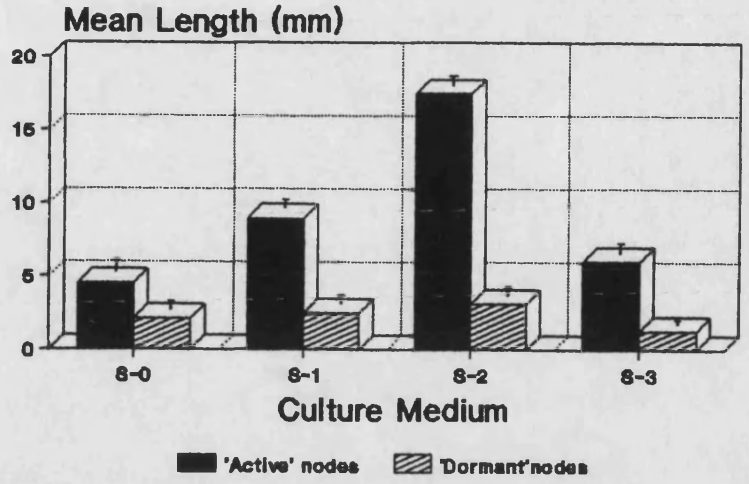


Fig 29(a). Mean lengths of axillary shoots of 'active' and 'dormant' nodes on four combinations of kinetin and BAP (n = 10).

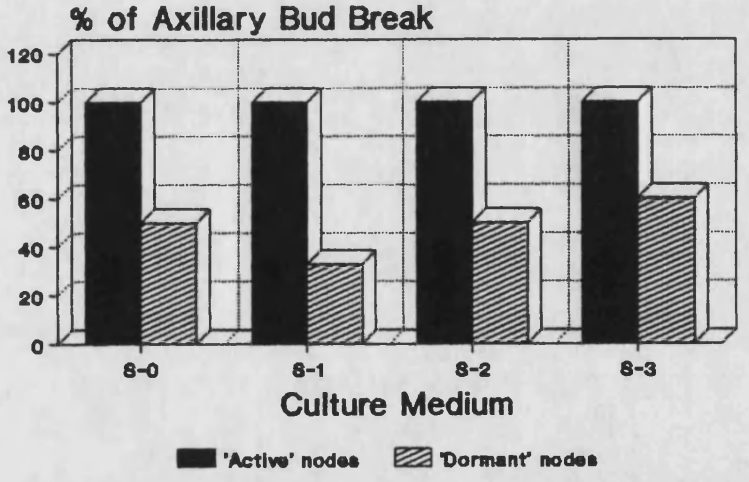


Fig 29(b). Percentage of axillary bud break of 'active' and 'dormant' nodes on four combinations of kinetin and BAP (n = 10).

As Fig 29(a) shows, the mean length of axillary buds of 'dormant' nodes, was smaller than that of the 'active' nodes. In fact, the figures of 'dormant' nodes after 4 weeks of culture, are

based only on 3-5 cultures, because the others were dead between 8-12 weeks showing no growth at all. The effect of the growth hormones on the length of the axillary buds of 'dormant' nodes was not very clear, because they were all very small. Some difference was observed between the media, on the elongation of axillary shoots of 'active' nodes, indicating that S-2 medium was the best.

The percentage axillary bud break of the 2 types of nodes, too, showed that the 'active' nodes were better explants. Up to 8 weeks, those on S-0 and S-1 media gave 90% axillary bud break, but at the end of 12 weeks all 4 media showed 100% axillary bud break. With 'dormant' nodes, the percentage axillary bud break remained around 50%, and no improvement was observed with time up to 16 weeks.

No leaf growth at all, was observed on the axillary buds of 'dormant' nodes; they were too small to produce leaves. With 'active' nodes, the axillary shoots produced on S-1, S-2 and S-3 media showed leaf growth, but this was not seen on those grown on S-0 medium. Therefore, in the later experiments, only 'active' nodes were used unless otherwise stated.

(c). The Effect of the Position of the Node.

This experiment was carried out in 2 parts. In the first part, the entire shoot was cut into 4 cm pieces starting from the top, the top 4 cm being the shoot tip labelled as 0 (Fig 30).

In this way, some nodes contained only one axillary bud on them, but most of the nodes carried more than one. Clones RRIC 110 and PB 86 were used to take explants. Six groups of explants labelled from 0 to 5, were compared for the axillary shoot growth. The medium contained half strength M&S salts, 2% sucrose, 100 ppm PVP and no growth hormones. Cultures were initiated in Petri dishes and then transferred into 100 ml jars. There were 10 explants from each node type, and cultures were transferred onto fresh media every four weeks.

The mean lengths of axillary shoots of each type of nodes up to 16 weeks of culture, are shown in Figure 31 (see also Appendix 17).

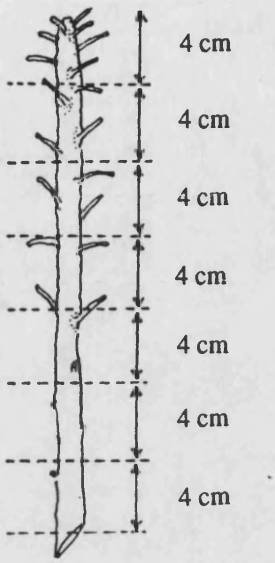


Fig 30. A shoot divided into equal size nodes.

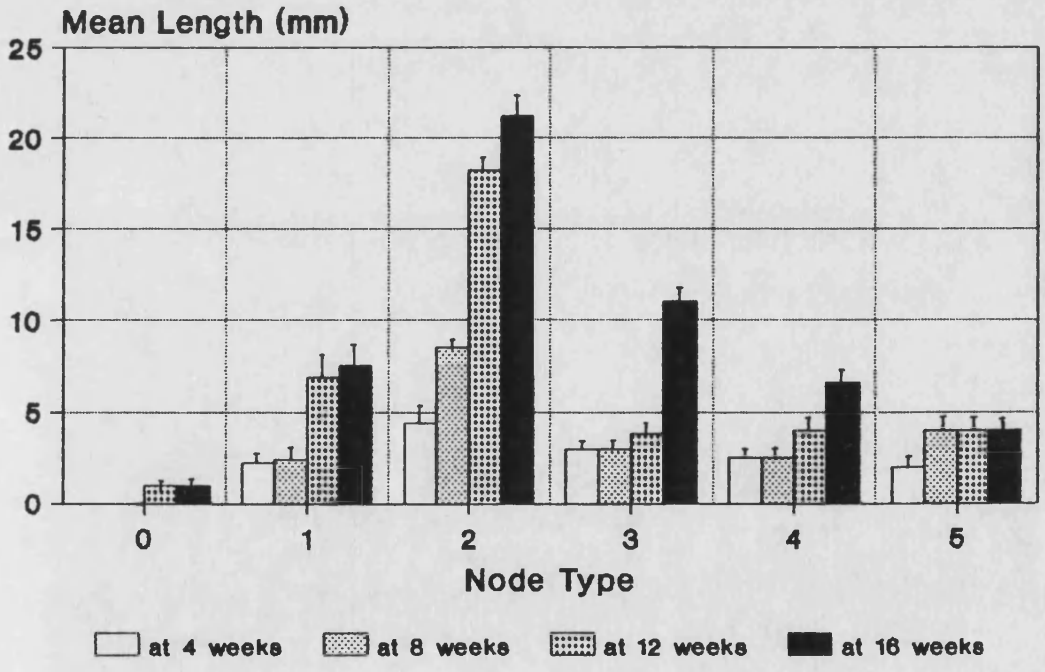


Figure 31. Mean lengths of axillary shoots of nodal explants labelled from 0 to 5 according to their position (n=10).

As shown in Figure 31, the shoot tips did not show any axillary shoot elongation. The nodal explants numbered as 1 and 2, were the best for axillary shoot elongation. In fact, the mean shoot elongation of node number 2 was about 3 times that of node number 1. From node number 3, the elongation of axillary shoots started to decrease down along the shoot. The contamination rate too was very high in these nodes. In most of the cases, the last 2 node numbers contained 'dormant' nodes as explained in the previous section of this experiment.

Leaf growth was observed in the axillary shoots of the second and the third 4 cm pieces. According to the results, the best nodes were those obtained in the second and the third 4 cm of the shoot.

The effect of the position of the node on axillary shoot growth was tested again, but this time, each axil was numbered (Figure 32) instead of equal size stem pieces used in the previous experiment.

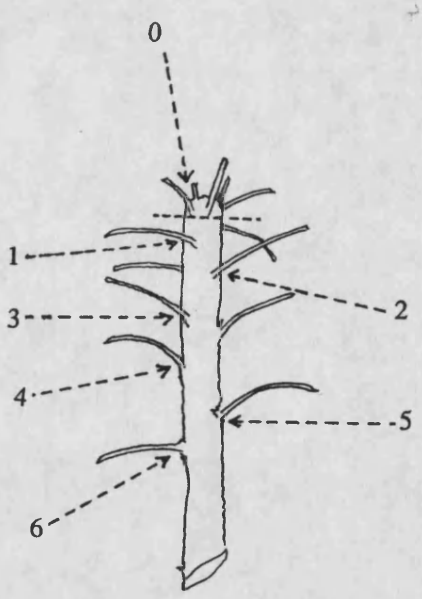


Fig 32. The 'active' nodes of a shoot numbered from 0 to 6.

In this experiment the node size varied from 0.5 cm to 2 cm, because at the shoot tip, internodal lengths were small which expanded down along the shoot. Here, only the active nodes (nodes with a leaf attached to them) were used. The top 5 mm of the shoot (shoot tip) was numbered as 0. The whole shoots were sterilized and then separated into nodes and cultured onto a hormone free medium. Cultures were transferred to fresh media every 4 weeks.

The mean lengths of axillary shoots up to 16 weeks of culture, are shown in Figure 33 (see also Appendix 18).

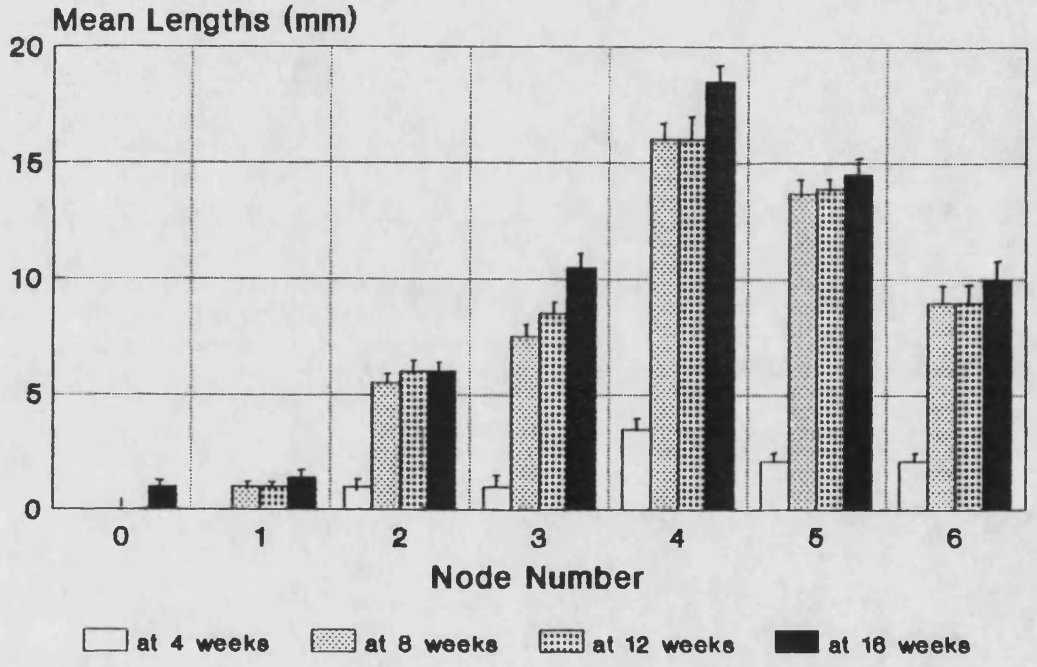


Fig 33. Mean lengths of axillary shoots of nodes numbered from 0 to 6 (n = 9).

This experiment was later repeated, and the same results were obtained as in this experiment. The lengths of the axillary shoots, increased from the apex up to the third or fourth node and then started to decrease. In general, leaf growth was observed in axillary shoots which were more than 10 mm long.

The elongated axillary shoots were cut into nodes and cultured onto a medium containing S-2 hormones (kinetin 7.5, BAP 3.75 and NAA 0.2 ppm). The newly formed nodes carried at least five or six axillary buds in 1 cm size piece. They all grew out, but remained less than 2 mm in length.

4.1.3. The Effect of the Basic Medium.

Only two basic media were compared, M&S and WPM, but three experiments were carried out along with three other variables.

(a). Comparison of M&S and WPM Media with Two Types of Nodes.

Nodal explants of clone RRIC 100 were used in this experiment. Although the 'dormant' nodes were found to be ineffective in axillary shoot proliferation, they were used again, because only M&S medium was used when they were first compared with 'active' nodes. M&S medium was made at half strength and WPM at full strength. Sucrose was supplied at 2% and PVP at 100 ppm. Media were prepared in Petri dishes for the first passage. They contained no growth hormones. After 4 weeks S-2 hormones were introduced and media were prepared in 100 ml jars. There were 15 replicates.

Results at 12 weeks of culture, are shown in Figure 34 (see also Appendix 19).

As shown in Figure 33, dormant nodes showed no better results in WPM medium. Contamination rate was higher with these nodes compared to that of active nodes. With active nodes, WPM medium was slightly better for elongation of axillary shoots. As far as the shoot quality was concerned, there was a significant difference between two media, as observed with juvenile materials. Axillary shoots grown on WPM medium were dark green and showed good leaf growth, while those grown on M&S medium were pale green and contained very small leaves. The survival rate was very low in M&S medium.

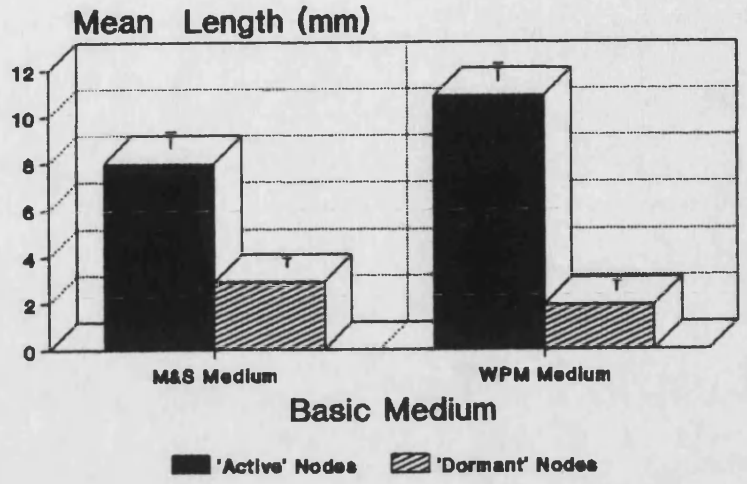


Fig 34. Mean lengths of axillary shoots of 'active' and 'dormant' nodes on M&S and WPM media (n=15).

After about 16 weeks of culture, axillary shoots were cut into nodes and cultured onto fresh media of the same composition. Those cultured onto M&S medium, did not survive beyond 20-24 weeks of culture. The nodes on WPM medium showed axillary bud break, but there was no substantial elongation, as observed with juvenile materials.

(b). Comparison of M&S and WPM Media with 3 Levels of Sucrose.

This experiment has been explained in Chapter 3 for juvenile materials of *Hevea* (Section 3.1.4). Media were prepared as for juvenile materials, but the hormones used here was S-2 (Kinetin 7.5, BAP 3.75 and NAA .2 ppm).

	2%	4%	8%
WPM	S-5	S-6	S-7
M&S	S-8	S-9	S-10

Mean lengths of axillary shoots produced in each medium, are shown in Figure 35 (see also Appendix 20).

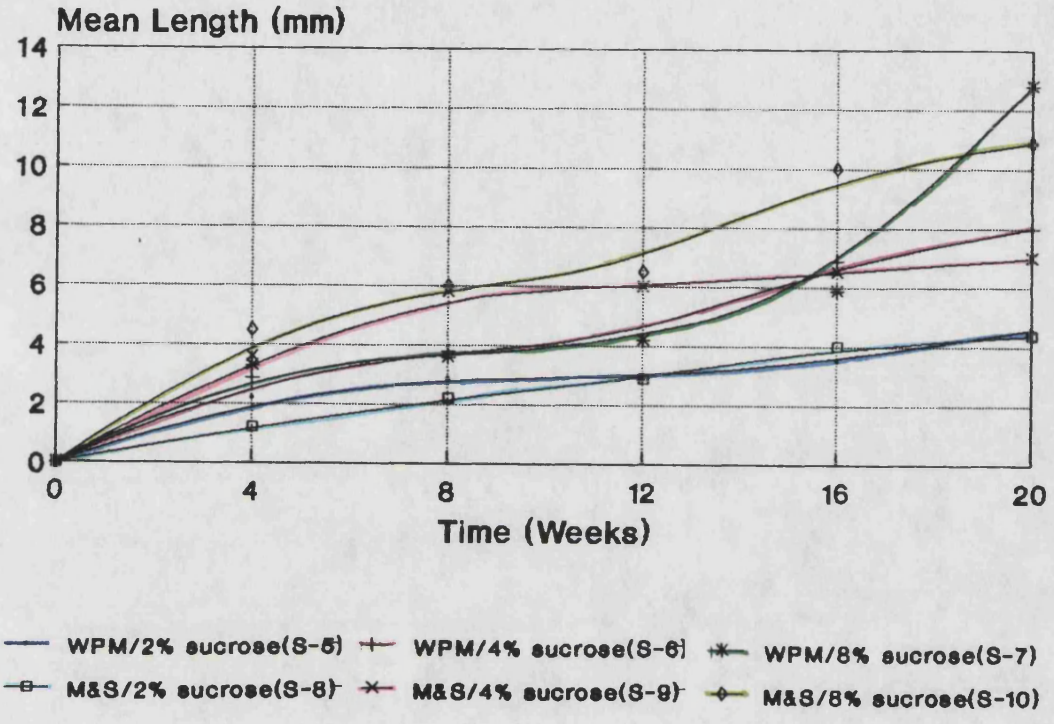


Fig 35. The mean lengths of axillary shoots on M&S and WPM media with 3 levels of sucrose (n=12).

As shown in Fig 35, the effect of sucrose was more prominent than that of basic medium on axillary shoot elongation. The axillary shoots grown on WPM media, were healthy looking and green than those grown on M&S media. As explained for juvenile materials, the most important factor was the higher survival rate of the cultures on WPM media. This was true with all 3 levels of sucrose. Better leaf growth was maintained in WPM media throughout the experiment. The mean lengths of axillary shoots increased with the increasing amount of sucrose in the medium. At 20 weeks of culture, elongated primary axillary shoots on WPM media were cut into nodes

and cultured onto same fresh media. Sucrose at 2% showed the least growth, and no difference was observed between the growth of secondary axillary shoots on 4% and 8% sucrose. Therefore, 4% sucrose was used in later experiments.

(c). Comparison of WPM and M&S Media with Two Types of Cytokinins.

This experiment too, has been carried out with nodal explants of juvenile origin, in Chapter 3 (Section 3.1.4.b). But, the amount of growth hormones used were different, and the three media were prepared as follows. 4% sucrose was used with solid media in 100 ml jars. BAP and NAA each at 0.2 ppm, were also present in thidiazuron containing media.

1. M&S + Thidiazuron (0.002 ppm)
2. WPM + Thidiazuron (0.002 ppm)
3. WPM + S-2 hormones (kinetin 7.5, BAP 3.75 and NAA 0.2 ppm)

Cultures were transferred onto fresh media of the same composition every 4 weeks. Sub division was carried out only when the axillary shoots or clusters of buds were large enough to seperate at least into two explants. The first subdivision of single axillary shoots grown on kinetin and BAP medium (S-2) was carried out at 24 weeks. Even after 32 weeks the mean number of propagules was only 1.7 due to their very slow expansion.

However, on thidiazuron containing media a different response was observed. No shoot elongation was observed as in S-2 medium, but, bud proliferation was high (Plate 14 b). On some cultures 4-8 buds were present but, the response was not uniform. Moreover, these buds did not survive, if seperated into individuals. But the explants with many buds could be subcultured and proliferated without shoot elongation. However, due to the high variation among the explants, some cultures had only a few buds which made the proliferation rate low.

The mean number of propagules produced on three media are given in the Table 7 (see also appendix 22).

Subculture time (weeks)	Culture medium		
	WPM/S-2	M&S/Thi.	WPM/Thi.
4	1	1	1
8	1	1	1
12	1	1	1
16	1	1.3	1.5
20	1	1.3	1.6
24	1.6	1.5	2.4
28	1.6	3.0	3.5
32	1.7	3.2	3.8

Table 7 The mean number of explants produced on three media (n=10).

The number of explants produced in the presence of thidiazuron was higher than that observed with kinetin and BAP. As far as the quality of the shoots were concerned, those produced on thidiazuron containing media were clusters of buds with very compact leaves (Plate 14 b). Compared to juvenile materials the growth of these clusters was very slow and uneven. No axillary shoot elongation was observed in any of the cultures.

Attempts were made to induce elongation of buds by transferring them onto hormone free media, by incorporating GA_3 into culture media (only 16 ppm was tested) and by transferring the clusters of buds onto rooting media containing activated charcoal(RM-5). Neither rooting nor elongation of axillary buds was obtained by any of the treatments.

The mean lengths of the axillary shoots produced are given in Fig 36 (see also appendix 21). With clusters of buds, the mean length measured was the hight of the clusters.

Some of the primary axillary shoots produced on S-2 medium (Plate 14 a) could be divided into two nodes and due to the very slow growth, there was hardly any proliferation on this medium (Fig 37 c). See also Appendix 22.

On the other hand, the clusters of buds produced on thidiazuron containing media could be proliferated with a propagule double time of 8-9 weeks (Fig 37 a & b). See also Appendix 22.

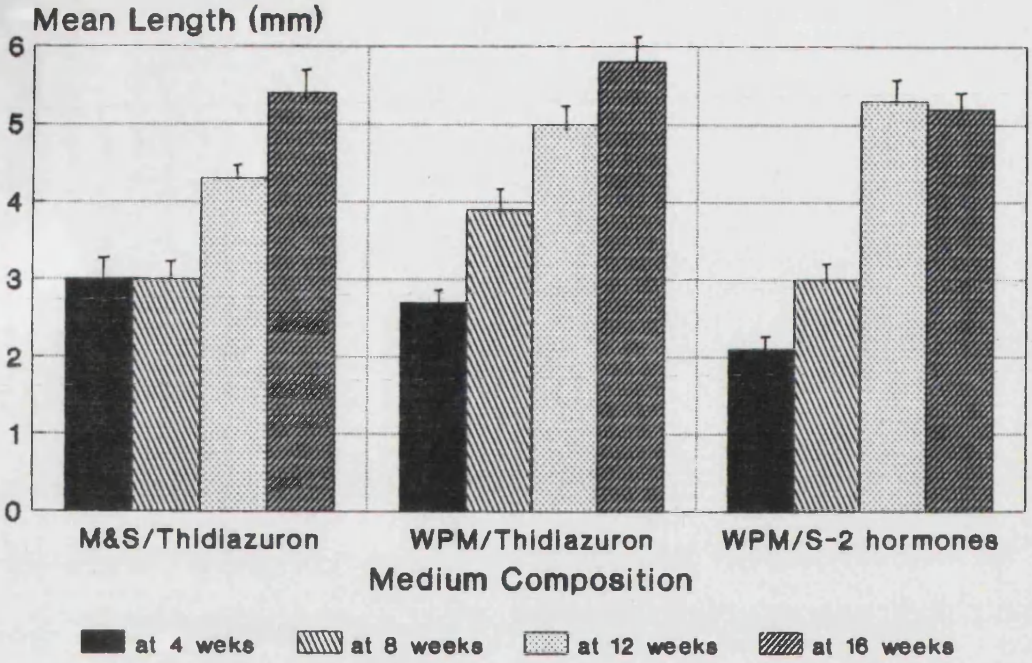


Fig 36. Mean lengths of axillary shoots on M&S and WPM media in the presence of thidiazuron and, a comparison of thidiazuron and conventional hormones with WPM medium (n=10).

Single axillary shoot growth was observed in S-2 medium. But, in both thidiazuron containing media, multiple axillary bud growth was observed in about 60% of the cultures. Compared to juvenile materials, the growth of these were very slow. Good leaf growth was observed in thidiazuron containing media. Although multiple axillary buds were induced on mature nodes, they were only clusters of very compact buds (Plate 14).

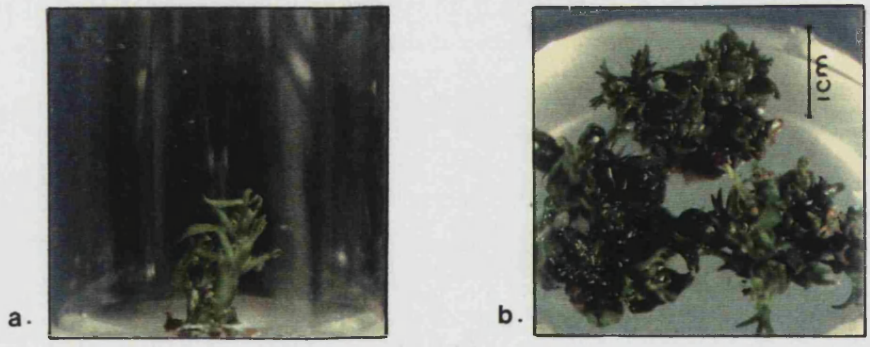


Plate 14(a). Single axillary shoots produced on S-2 hormones and **(b)** clusters of buds produced in the presence of thidiazuron. Results after 20 weeks of culture.

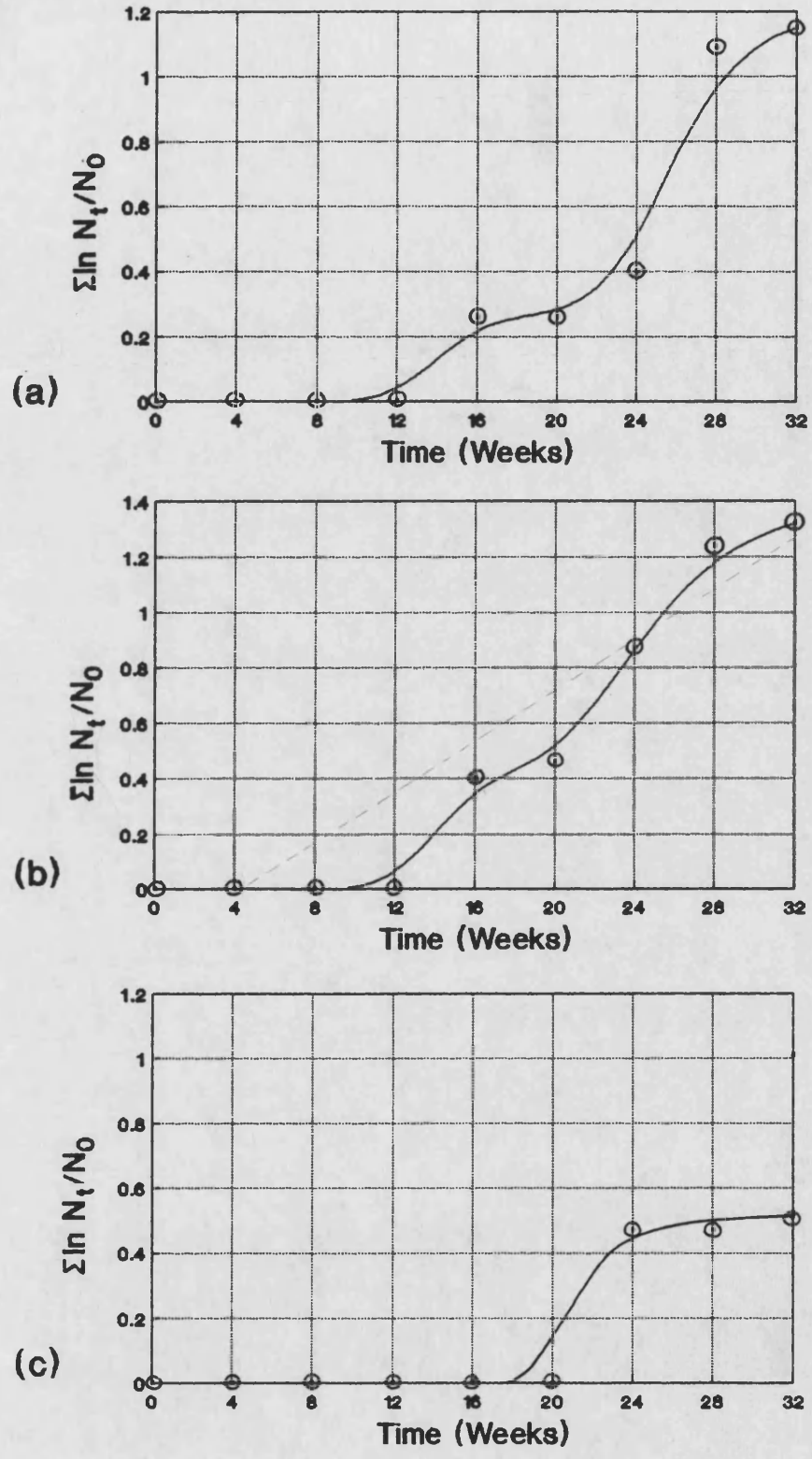


Fig 37. Axillary shoot proliferation of nodal explants (a) M&S/Thidiazuron(0.002 ppm), (b) WPM/Thidiazuron(0.002 ppm) & (c) WPM/S-2 hormones. (n = 10).

Therefore, in later passages, thidiazuron showed no advantage over conventional hormones because of the very little elongation observed in the axillary buds produced. Shoot doubling times were not calculated, because the growth rate and the quality of the shoots on non of the media were satisfactory. However, the survival rate and the shoot quality were always high in WPM media.

4.1.4. The Effect of the Level of Sucrose on Axillary Shoot Growth.

Five concentrations of sucrose (0, 2, 4, 6, 8 and 10%), were compared in this experiment. The basic medium used was WPM supplied with PVP at 100 ppm. Thidiazuron 0.002, BAP 0.2 and NAA 0.2 ppm were used as hormones. This medium was used in the previous experiment with some success. Media were prepared in Petri dishes for the first passage and then in jars. There were 10 replicates. Results up to 16 weeks of culture, are given in Fig 38 (see also Appendix 23).

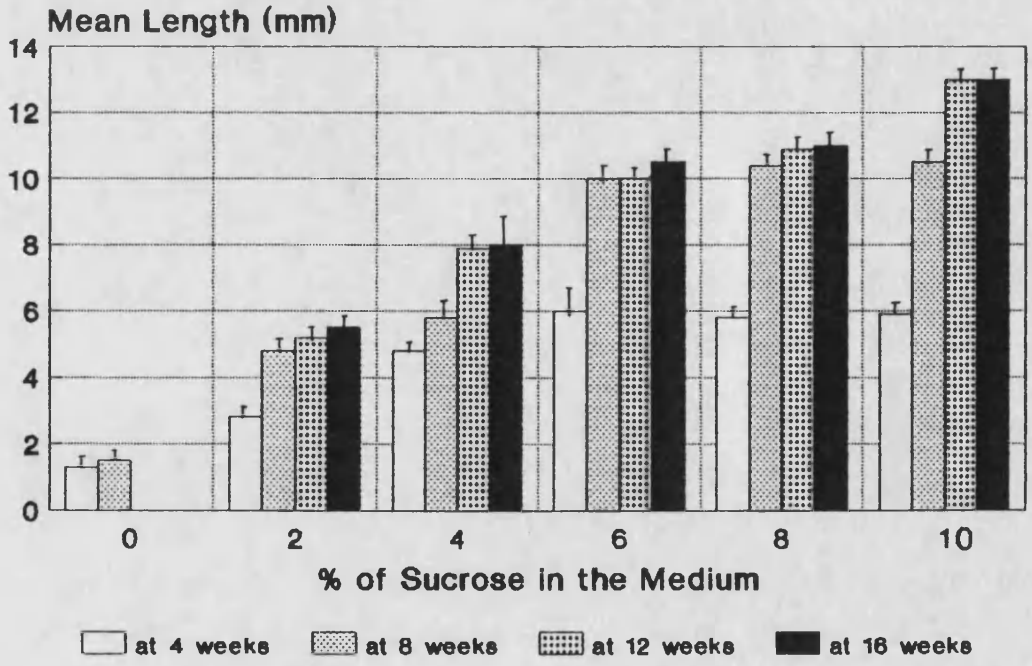


Fig 38. Mean lengths of axillary shoots grown at five levels of sucrose (n=10). Cultures were recultured every 4 weeks.

As expected, cultures grown on sucrose free medium did not survive beyond 8 weeks of culture. The mean length of axillary buds produced, at the end of 8 weeks was only 1.5 mm, which was very low compared to the others. The length of axillary shoots increased with the increasing amount of sucrose in the medium from 2% to 10% (Plate 15).

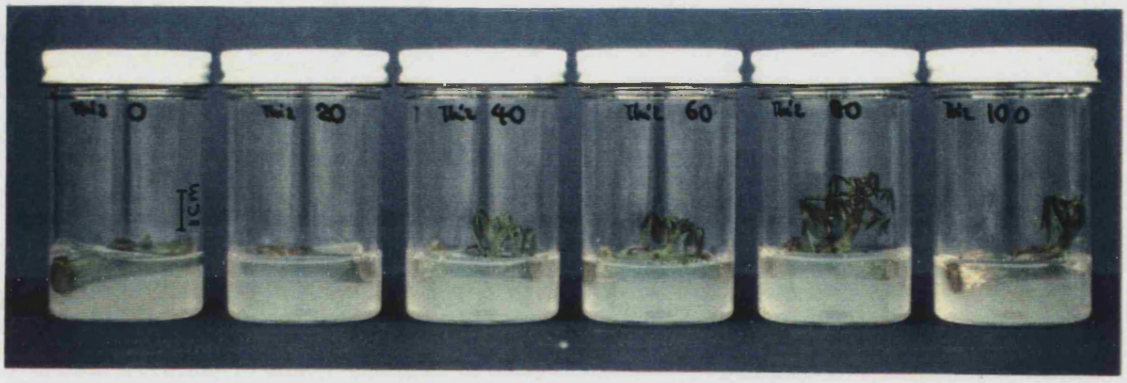


Plate 15. Axillary shoot growth of nodal explants grown at five concentrations of sucrose (n=15).

As Plate 15 shows, the difference observed between the mean lengths of axillary shoots, produced at different levels of sucrose, was very small, because the maximum length observed at 10% sucrose was only about 13 mm.

About 75% of the cultures on 6, 8 and 10% produced only single axillary shoots, although thidiazuron was used in the culture media. The reason might be the quick elongation of axillary shoots at high sucrose before the buds were induced to produce multiple buds. At 2% and 4% sucrose, about 50% of the cultures showed multiple buds. But compared to juvenile materials, the growth was very poor and slow.

Considering the shoot quality and the leaf growth of the axillary shoots produced, both 6% and 8% sucrose were equally good. After 16 weeks of culture, the axillary shoots were divided into nodes and cultured onto same fresh media. Each node contained 1-5 axillary buds on them.

The mean number of propagules produced on 2%, 4%, 6%, 8% and 10% sucrose containing media, were 1, 1.5, 2.1, 2.0 and 1.5 respectively. Again, these figures were directly related to the lengths of the primary axillary shoots produced.

Unfortunately, this experiment could not be continued, because the cultures were heated up and died due to a growth cabinet failure.

Although the number of propagules produced at 4, 6, 8 and 10 % levels, were not very different from each other, 6% was chosen to use in later experiments, because of the good quality of the secondary axillary shoots produced and the good leaf growth observed at this concentration of sucrose.

4.1.5. The Effect of Exogenous hormones on axillary shoot growth.

(a). BAP as the Only Cytokinin.

WPM medium with 6% sucrose was used in Petri dishes for the first passage and after that in 100 ml jars. BAP was used at 2, 4, 8 and 16 ppm, with NAA at 0.2 ppm. Results after 12 weeks of culture are shown in Figure 39 (see also Appendix 24).

The results obtained showed, that neither the highest nor the lowest BAP containing media were beneficial for the elongation of axillary buds. Both 4 ppm and 8 ppm gave the same mean lengths of axillary shoots. The leaf growth was highest with 8 ppm BAP. Even at 16 weeks, the maximum length observed was 1 cm on media containing either 4 ppm or 8 ppm BAP.

At 16 weeks, the apices were removed from the axillary shoots and cultured onto same media. Axillary bud break was observed on decapitated shoots after 4-6 weeks of culture. In some cases only one axillary bud (the first axillary bud from the top) gave about 8 to 10 mm elongations, but no leaf growth was seen on these shoots.

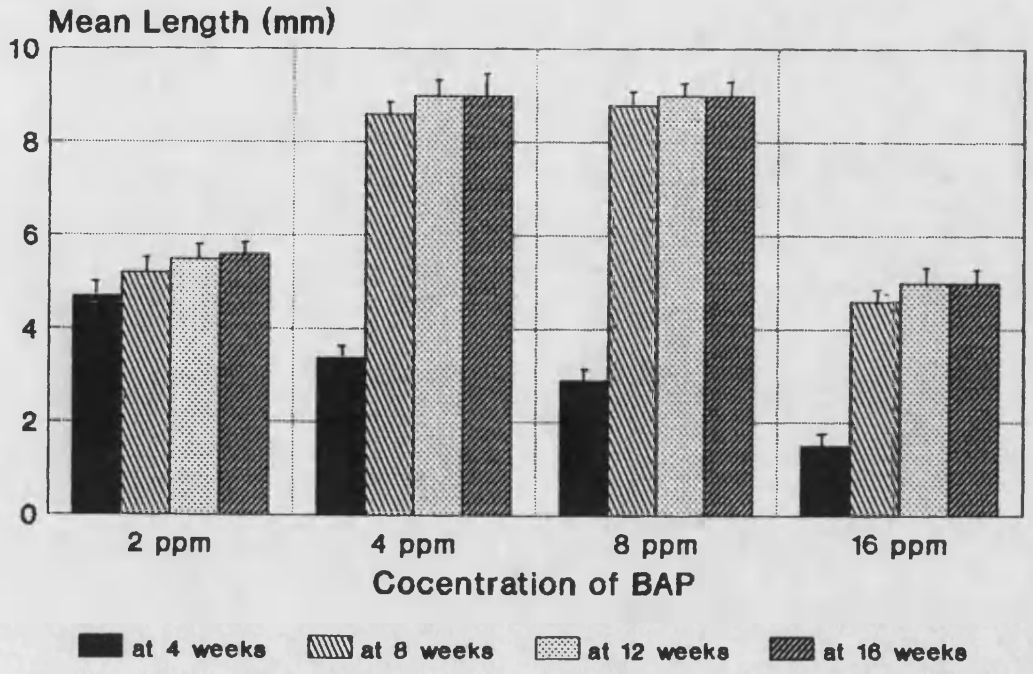


Fig 39. Mean lengths of axillary shoots grown at four levels of BAP (n=12).

(b). Combinations of kinetin and BAP.

The same four combinations of kinetin and BAP, designated as S-0, S-1, S-2 and S-3, were used here again. The compositions of the media are given on page 49 in Chapter 3. The mean lengths of axillary shoots on four media up to 16 weeks of culture, are given in Fig 40 (see also Appendix 25).

As Figure 40 shows, the maximum axillary shoot length was observed in S-2 medium. Those produced on S-3 medium had very small internodal lengths. Control medium showed the poorest axillary shoot growth which was less than 5 mm long, while S-1 medium showed intermediate elongation.

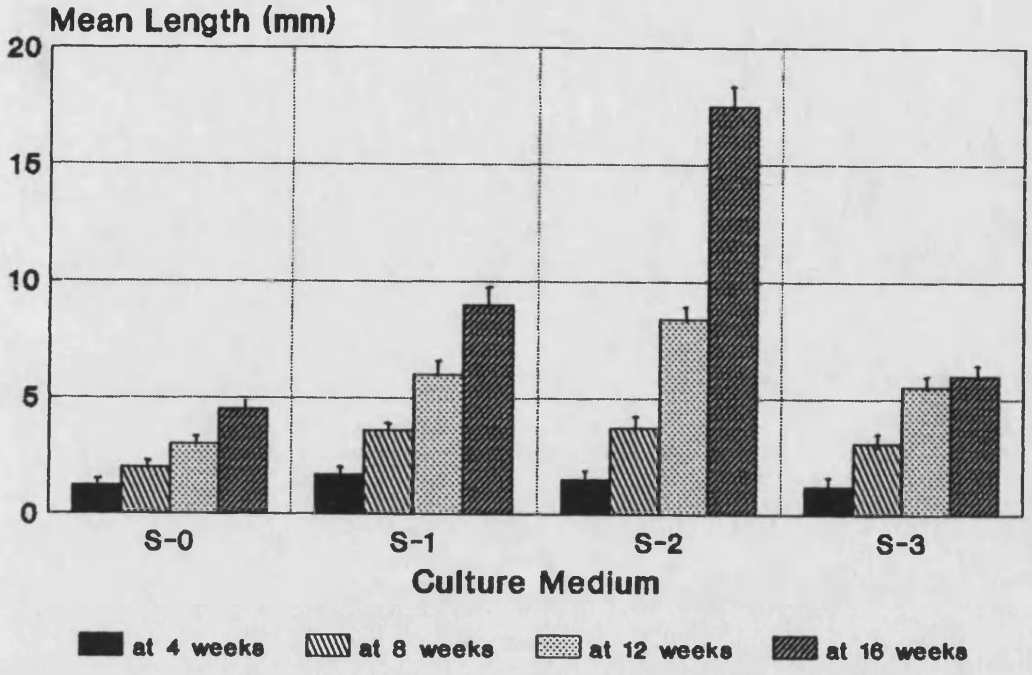


Fig 40. Mean lengths of axillary shoots grown on four combinations of kinetin and BAP. (n=11).

The leaf growth too, was highest on S-2 medium. The laves produced on S-3 medium were pale green and small. Axillary shoots on S-0 medium had no leaves, and there were some leaves on those on S-1 medium.

Attempts were made to continue the axillary shoot proliferation using nodal explants. But, as experienced with juvenile material, the elongation and the growth of axillary shoots after the secondary nodes, was very low compared to the primary axillary shoots.

(c). 2iP Containing media.

Again in this experiment, the only alteration made was the type of cytokinin in the medium. Four levels of 2iP were tested, 2, 4, 8 and 16, with a control medium containing no hormones.

NAA was present at 0.2 ppm in all the media.

Results at the end of the 16 weeks, are given in Figure 41 (see also Appendix 26).

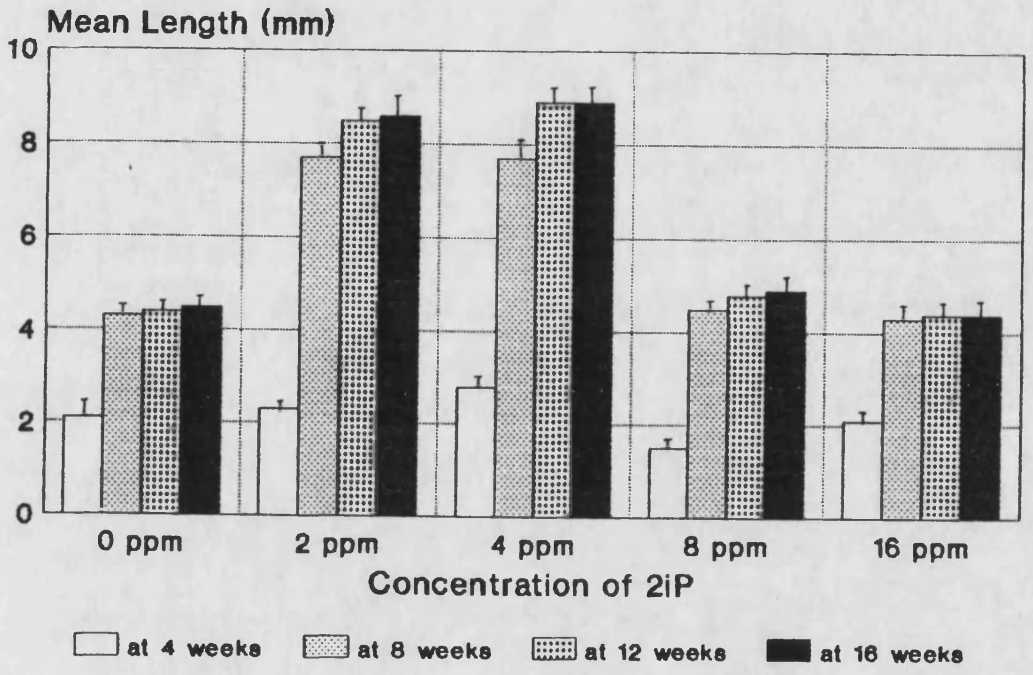


Fig 41. The mean lengths of axillary shoots grown at four levels of 2iP (n=11).

The only advantage of using 2iP instead of BAP, was the good leaf growth observed in 2iP containing media throughout the experiment. The maximum axillary shoot elongation observed was 1 cm which was not substantial to divide into nodes. However, axillary shoots were transferred onto fresh media, with their apices removed. Secondary axillary buds of about 1-2 mm were observed, but no further development occurred for another 12 weeks.

(d). Thidiazuron as a Cytokinin.

In this experiment, a range of concentration of thidiazuron was tested. Thidiazuron at 0, 0.002, 0.01, 0.05, 0.25, 1.25 and 6.25 ppm were used with 0.2 ppm NAA. BAP was not used in this experiment. Solid WPM medium, supplied with 6% sucrose and 100 ppm PVP, was made in Petri dishes, and there were 10 replicates.

The mean lengths of axillary shoots for the first 8 weeks of culture, are given below.

Concentration of thidiazuron (ppm)

weeks	0	0.002	0.01	0.05	0.25	1.25	6.25
4	5.8	3.2	2.5	1.9	2.3	1.9	1.3
8	6.5	7.0	4.6	3.9	3.5	3.0	1.6

Table 8. Mean lengths of axillary shoots of nodal explants at five levels of thidiazuron (n=10).

Axillary bud break occurred in most of the cultures. The maximum shoot elongation was seen in the cultures on control medium and at 0.002 ppm thidiazuron. The length of the axillary buds decreased with the increase of thidiazuron from 0 to 6.25 ppm.

After 8-10 weeks, the cultures on media containing more than 0.25 ppm thidiazuron, started to turn brown, and in 2-3 weeks most of the cultures died leaving only 2-3 replicates in each medium. The remaining cultures showed initiation of multiple axillary buds. But, compared to juvenile materials the growth of these were very slow.

Another experiment was carried out with thidiazuron since the previous one was not very successful. In this experiment the following media were tried.

hormone	Thi.1	Thi.2	Thi.3	Thi.4	Thi.5	Thi.6	Thi.7
Thidia:	0.1	0.002	1.0	0.1	0.1	0.001	0.1
BAP	0.2	0.2	-	0.1	0.5	0.2	-
IAA	0.05	-	-	-	-	-	-
NAA	-	0.2	0.2	0.1	-	0.2	0.1

WPM solid medium supplied with 6% sucrose was used first in Petri dishes and then in 100 ml jars. The mean lengths of axillary buds produced are given in Table 10.

weeks	Thi.1	Thi.2	Thi.3	Thi.4	Thi.5	Thi.6	Thi.7
4	0	0	0	0	0	0	0
8	1.2	1.9	1.8	1.0	1.8	2.0	1.5
12	1.2	1.9	1.9	1.8	1.9	2.3	1.8
16	1.3	4.4	2.3	2.0	2.0	2.9	2.4
20	1.3	4.8	2.9	2.4	2.4	3.1	2.5
24	1.4	5.1	3.1	2.7	2.7	4.4	2.5

Table 9. Mean lengths of axillary shoots produced on thidiazuron containing media (n=11).

All thidiazuron containing media except Thi.6, showed some multiple axillary buds after about 16-20 weeks of culture. The elongation of these axillary buds were very poor, about 3-4 mm. Nodal explants grown on Thi.6, produced only single axillary shoots.

(e). Soaking of Explants in Hormone Solutions.

The following three soaking treatments were used in this experiment.

I... BAP 10 ppm + IBA 5 ppm for 2 hours.

II.. BAP 10 ppm + NAA 5 ppm for 2 hours.

III. BAP 10 ppm + NAA 5 ppm for 4 hours.

Hormone free solid WPM medium supplied with 6% sucrose, 5% charcoal and 100 ppm PVP, was used in Petri dishes. After the second passage, media were prepared in 100 ml jars. Cultures were transferred onto fresh media every 4 weeks time. The mean lengths of axillary shoots are given in Figure 42 (see also Appendix 27).

Axillary bud break and elongation showed no great difference between treatments. Leaf growth of the cultures of the treatment I, was poor compared to that to the other two treatments. Callus growth was observed in all cultures.

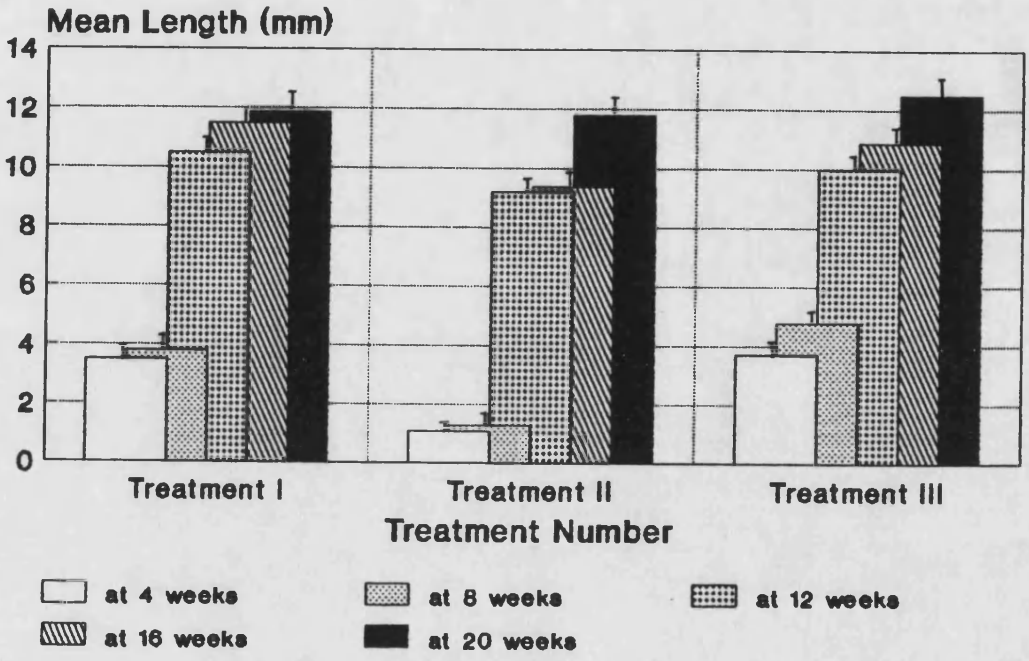


Fig 42. Mean lengths of axillary shoots of nodal explants of three soaking treatments (n=14).

About 40% of the cultures of treatment II, was dead at the end of the 20 weeks. Leaf growth was fine in the remaining cultures. Those of treatment 3 gave the maximum callusing. Leaf growth was poor, but the maximum axillary shoot elongation was noticed in this treatment.

Cultures of all three treatments, showed contaminations in about 25-40% of them. The axillary shoot elongation observed was as good as that of S-2 hormones (kinetin 7.5, BAP 3.75 and NAA 0.2 ppm). At the end of 20 weeks, there were not enough replicates in each treatment, and the experiment was therefore terminated.

4.2. Discussion

The explants used for *in vitro* culture of most tropical trees, were from embryos, seedlings or buds of mature trees. In certain plants, the seedling or juvenile materials were first tried or grown successfully, and on the experience gained, the explants of mature origin trees were experimented. This method, even though ambiguous, gave satisfactory results in some elite and high yielding trees or their varieties.

For some other plants, micropropagation from explants of mature trees, if at all possible, may require a cultural regime that is considerably different from that required for juvenile explants. Even if the explants from the mature trees were obtained from material collected from the best position and at the best time, to provide cells as juvenile as possible, these cells are often not as organogenetically competent as those from juvenile origin.

The first, among the many problems found with mature material, was the culture establishment. This was mainly due to the difficulties in surface sterilization and the high content of phenolic browning. These two problems have been mentioned by almost every body who worked with mature origin materials.

In the present studies, shoot tips and nodes collected from mature materials belonging to five selected clones of *Hevea*, were used. As explained earlier in the results section, neither shoot tips nor nodes could successfully be surface sterilized with NaOCl. The main reason for this was the high content of phenolic compounds in the tissues, which easily get aggravated by the treatment of NaOCl.

Once phenolic compounds are oxidised, they produce quinone compounds which are brown. These diffuse gradually into the medium, and inhibit activities of other enzymes, and then damage the whole explant. Factors affecting explant browning are complex. In the plant, as plant species, genotype, locations of explants and physiological conditions are different, the degree of browning may vary. Also there are so many other factors affecting on the browning of explants.

The strength of the basic medium, had an effect on the extent of browning of *Hevea*. M&S medium at full, 1/2 and 1/4 strength, were tested and found that browning was higher at full strength than the other two. But there was no visible difference between the 1/2 M&S and 1/4 M&S media. Anderson (1975) also reported that the browning of *Rhododendron* shoots, could be overcome by reducing the nitrogen-containing compounds in the M&S medium to one half.

One of the reasons that woody plants grow better in low salt containing media, might be the increase production of phenolics at high salt levels, although these were not always visible to naked eye. Most of the woody plants were grown successfully on lower salt containing media than on M&S as discussed in Chapter 3. Incubation of shoot tips or nodes in the dark at low temperatures, was not effective to reduce browning of clonal origin explants in the present studies. Barghchi and Alderson (1983) also reported that there was no benefit from incubation *pistacia* shoot tips in the dark to prevent or reduce phenolic browning. More frequent subcultures or treatments with charcoal or PVP, have been suggested to be useful to overcome this problem (Barghchi and Alderson, 1985). But, reduction of light levels was an effective method to reduce phenolic exudation of garlic meristems. According to Hu and Wang (1983), cultures illuminated under 150 lux during the first month showed only 4% browning, while 18% browning occurred under 500 lux. In order to reduce browning, Adams et al (1979) conducted the culture initiation stage in total darkness.

Use of antioxidents to control browning, is a commonly used method for some species with success. In the present studies, soaking of explants in an antioxident solution, increased the extent of browning. Antioxidents used were ascorbic acid and citric acid at 100 ppm and 150 ppm respectively. By incorporating the same mixture of antioxidents into the solid medium, slowed down the process of browning. But, in the liquid medium no effect at all was observed.

McComb and Newton (1981) tested several antioxidents in order to control polyphenolic exudates which turned the entire medium dark blue. Ascorbic acid 1 mM, PVP 0.1 mM, dithiothreitol 1 mM and bovin serum albumen 0.25 ppm were all ineffective to reduce the

browning, but eventually some clones were selected as they produced less phenolics in standard media. In the present studies, water soluble PVP (M.W. 44000) incorporated at 100 ppm into growth medium, was very effective to reduce phenolic browning of mature *Hevea* shoots. McComb and Newton (1981) have not described the type of PVP used or the method, to compare with the present results.

Activated charcoal have been effective to reduce phenolic browning in some plant species. This might have been caused by the adsorption of substances that were released from the explants into the culture medium by charcoal. Preil and Engelhardt (1977) observed positive effects of charcoal, when added to the medium at the beginning of the culture initiation phase, but this prevented the formation of several shoots per explant. This may have been a result of the adsorption of BAP by the activated charcoal. Tisserat (1979) showed that addition of activated charcoal enhanced the percentage of explant survival, and organogenesis of date palm. Increasing concentration from 0.1 to 0.3%, increased the survival rate and organogenesis equally. PVP showed no beneficial effect on reducing browning of date palm.

Carron and Enjalric (1982) used activated charcoal at 5% for juvenile nodes of *Hevea*, to obtain better shoot growth. In fact, juvenile materials of *Hevea* do not contain phenolics at harmful levels. However, in later experiments they did not use charcoal in the medium (Carron et al,1989). Presence of activated charcoal in the medium generally supported the growth of leafy shoots.

Frequent transfer of cultures onto fresh media, was very effective to reduce browning of mature *Hevea* shoots, in the current experiments. In this method, cultures had to be transferred frequently enough in order to obtain positive results. In the present work, they were transferred at least five times within the first day, and then daily up to a week and weekly up to one month. Browning was observed throughout the first week but in reducing amounts.

Ripley and Preece (1986) transferred the explants of *Euphorbia lathyris* to fresh media at days 1, 3 and 5, and obtained a decrease of dark lethal exudates to non lethal levels. The

browning problem of *Rhododendron* shoot tips, was overcome by a series of frequent transfers to fresh media (Ettinger and Preece, 1983). But, the frequency of transfer required was not stated. Anderson (1975) transferred *Rhododendron* shoot tips every three weeks to keep them alive in addition to the use of low salt medium.

Lloyd and McCown (1980) had to transfer the shoot tips of mountain laurel onto fresh media at 12 and 24 hours after the initial culturing, and then on a daily basis for about one week until medium decolouration was under control. Broome and Zimmerman (1978) controlled tissue and medium darkening of blackberry shoot tips by transferring explants onto fresh media one to two days after initial culturing. Transferring weekly was sufficient to minimize the inhibitory effects of polyphenols of fig shoot tips (Muriithi, et al, 1982). Frequent transfer of phenolic producing explants onto fresh media until they stopped producing them, seemed to be an effective method to control browning, although this method can be very time consuming and labour intensive.

Use of polyvinylpyrrolidon (PVP) was the most effective and efficient method found to control phenolic browning of *Hevea* in the current work. Since the molecular weight and the solubility can vary, it is necessary to choose the correct type of PVP. In the case of *Hevea*, water soluble PVP of which the molecular weight was 44000, efficiently reduced brown exudates. Insoluble PVP as a pretreatment or added to the culture medium, was not effective. Even water soluble PVP showed no beneficial effect as a pretreatment. PVP at 100 ppm in the medium, was as effective as 200 ppm, and therefore, the lower concentration was used in the media in later experiments.

Mascarenhas et al (1987) used insoluble PVP (M.W. 36000, crosslinked) at 0.75% with 2% sucrose, as a pre-wash for 30 minutes. *Aesculus* explants were agitated in a solution of 7% PVP for 2 hours in order to eliminate brown exudates (Hosier and Read, 1983). Christiansen and Fonnesbech (1975) found that more *Hamamelis* explants survived and developed with the increase of PVP concentration in the medium. They obtained best growth and development at 1% PVP.

PVP is a polymer and a strong hydrogen accepter, but cannot act as a hydrogen donor (Forest, 1969); this compound adsorbs phenols and inhibits catechol oxidase (a polyphenol oxidase). Sommers et al (1982) observed that PVP may have inhibited shoot proliferation of black walnut shoot tips, and that the medium with PVP was darker than the medium without PVP.

One among the several methods tried by Ziv and Halevy (1983) to control oxidative browning *Strelitzia reginae*, was the use of $HgCl_2$ (at 0.3%) to sterilize explants. They also reported a higher contamination rate of explants after soaking treatment with antioxidents.

The present finding of the reduction of phenolic browning further, as well as increasing the number of clean cultures by using $HgCl_2$ as the sterilant is in agreement with the work of Ziv and Halevy. $HgCl_2$ has been used by many other workers (Huang and Tan, 1990; Chen and Evans, 1990; Monteuuis et al, 1987; Muriithi, 1982) to sterilize shoot materials. Manzanera and Pardos (1990) used 0.1% NaOCl for 10 minutes to sterilize juvenile origin materials, but for the shoots of mature origin, 0.2% $HgCl_2$ was used for 2 minutes followed by two washes in 0.24% $CaCl_2$ and 0.1% ascorbic acid respectively. For the sterilization of terminal buds of teak, Mascarenhas (1987) used 0.15% $HgCl_2$ solution for ten minutes. Jelaska (1987) used a solution of 0.1% of $HgCl_2$ prepared in 50% ethanol and 0.3% Tween 20 for 10 minutes to sterilize lateral branches of incence-cedar. Prior to sterilization, cut surfaces of explants were sealed with hot wax.

Sealing the cut ends was tried in the present studies as well prior to using $HgCl_2$ or ethanol. All the cut ends were quickly dipped in hot wax in order to prevent absorption of $HgCl_2$ which could be toxic to the cultures. But later this method was successfully replaced by removing two 1 cm size pieces from either sides of the node after sterilization.

The success of the present work with PVP in reducing phenolic exudates, is partly due to the culture of explants horizontally. This allowed maximum mutual transport between the explant and the medium. The effect of PVP was very little, when the explants were cultured vertically, because of the requirement of fast adsorption of phenolic compounds by the PVP which is in the medium. Horizontal orientation must have been helpful for the explants to use the other

components of the culture medium too, such as growth substances, efficiently. Campbell and Tomas (1984) reported that the red clover shoot tips placed horizontally, produced significantly more shoots and a higher percentage of cultures of multiple shoots than those placed vertically in the media. Placing crown buds horizontally may have allowed more rapid growth of normally quiescent axillary buds on the explants. Such axillary buds may have been released from the apical dominance by a combination of the horizontal orientation and the cytokinins in the medium. Yang (1977) also obtained a higher production of shoots from the shoot tips placed horizontally than those placed vertically. But, this combination was not strong enough to overcome the apical dominance of *Hevea* shoot tips.

In the present studies, it was found that the extent of phenolic browning was also dependent on the explant type, physiological state and the clone of *Hevea*. Shoot tips showed more phenolic browning than nodal explants. This was true for juvenile material of *Hevea* too, although the amounts present were harmless and therefore negligible. Shoot tips and nodes collected at their actively growing state, contained more phenolics than those collected at their stationary state of growth. The extent of phenolic browning observed in the five clones was also different. This was determined only by the intensity of the colour. Chevre and Salessen (1987) also reported that different amounts of phenolic compounds present in different clones of chestnut, varied from no phenolics to very high. According to Chevere and Salessen the content of phenolic compounds seemed to be closely related to the physiological condition of the plant and to its genotype; these compounds seemed to play a part in organogenesis *in vivo* as well as *in vitro*.

To overcome the high rate of contamination found in *Hevea*, Carron et al (1985) used a mixture of antibiotics containing kenamycine 20 ppm, chlorotetracycline 10 ppm and chloramphenicol 60 ppm in the medium. Again in 1988 Enjalric and Carron recommended to use a mixture of antibiotics including a fungicide, gentamicin 20 ppm, kenamycin 20 ppm, chlorotetracycline 30 ppm, chloramphenicol 60 ppm, rifampicin 75 ppm and the fungicide, benomyl 750 ppm to spray on mother plants where explants were taken. At the beginning this

antibiotics and fungicide mixture was either incorporated into the medium, or used for soaking the plant material for 20 hours before culture was started. But, they found that the bud opening was greatly affected by either of the treatments.

Spraying mother plants with the same solution every two days for 15 days, before the excision of culture material, showed some improvements without affecting the axillary bud growth. Prior to sterilization, they brushed the surface of the explants in 95% ethanol which increased the number of aseptic cultures by about 30%.

They tested several sterilization treatments, NaOCl (11-22% chlorine), $HgCl_2$ (0.5-1 g/l) and H_2O_2 (60-110 vol), for 15 to 30 minutes with a pre wash in 95% ethanol for 3-4 minutes and found all ineffective to reduce contaminations.

Use of $HgCl_2$ was highly effective to reduce contamination rate in the present work, while improving the number of cultures with no phenolic browning. The concentration of $HgCl_2$ used by them was only 0.05-1%. In the present studies the use of 0.2% $HgCl_2$ for 10 minutes with a pre wash in 70% ethanol for 1 minute, resulted 100% clean cultures most of the time.

Pollock et al (1983), Bebrgh and Maene (1984) and Bastiaens et al (1983) reported that the use of antibiotics to control contaminations of cultures, were not very effective against the germs, but showed definite phytotoxicity. This conclusion was supported by the finding of Enjalric et al (1988) as explained by themselves above. On the other hand, Willson and Power (1989) reported of successful use of mixture of antibiotics in protoplast cultures of *Hevea*.

A few experiments on selection of explants were carried out with mature origin materials, as reported for juvenile materials in Chapter 3. The results obtained by comparing shoot tips and nodes, were similar to those obtained for juvenile materials. Presence of strong apical dominance was further evident. Barghchi and Alderson (1983) also noticed that the apical dominance was much stronger in explants from 2 year old trees than from seedlings, and removal of apical bud encouraged more axillary buds to develop into shoots. In *Hevea* removal of about 10 mm size

shoot apex was sufficient to remove apical dominance to some extent.

As explained in the Section 5.1.2(b), only the top half or 1/3 of the shoot beared leaves at each axil in *Hevea*. The axils in the bottom half contained no leaves attached to them. For the easy identification they were called as 'active' and 'dormant', active being the axils with a leaf attached to them. Nodes containing these two types were compared for their ability to produce axillary shoots. From the four combinations of kinetin and BAP tested, active nodes proved to be superior to dormant nodes. Axillary bud break on active nodes were 100% in all media including the control. With dormant nodes, the maximum percentage axillary bud break observed was 60% in the highest cytokinin containing medium and the maximum axillary shoot elongation was about 3 mm in all four media. With active nodes, maximum length of about 17 mm was observed. The length increased with the amount of exogenous hormones, but decreased at the highest level. There was no further growth of the axillary buds of dormant nodes, and they turned brown after 8-12 weeks of culture.

The effect of the position of the node was tested again, but this time the shoot was cut into equal size pieces starting from the top, the top 4 cm piece being the shoot tip numbered as 0. Although these were numbered from 0 to 5 the numbers did not represent each axil, because some nodes contained more than one axillary bud. As expected, shoot tip showed no axillary bud elongation. The mean length of axillary shoots of node number 3 was the highest. This again confirmed the presence of apical dominance in the apex, which decreased with the increasing distance from the apex. After node number 3 which had the least effect from the apex or which had the highest mean axillary shoot length, the axillary shoot lengths started to decrease. This may be due to the degree of dormancy or inactivity of the axillary buds gained with their age.

The next experiment towards the choice of explants, was carried out with nodes carrying single axils labelled from the apex. Node sizes varied from 0.5 to 2 cm. This experiment showed that mean axillary shoot lengths of node numbers 4 and 5, were higher than the rest. Node numbers 3 and 6 were the next, and after that the numbers 2, 1 and 0 (shoot tips) were in

descending order. All these were observed in hormone free media. Therefore, this observations were their natural potentials when detached from the tree. Generally, the content of endogenous growth substances seemed to vary with the position of the tissue on the parent plant and with environmental condition. Also the content of endogenous growth substances of the tissues vary with ontogenetic and physiological age of the tissues, and so each tissue has a different demand for exogenous growth substances. Cresswell et al (1982) reported the effect of shoot position of *Eucalyptus*, on the ability to produce roots. Best rooting occurred in nodes where a functional leaf was present. When nodes with very young leaves less than two thirds of the final size, or nodes from which the leaves had already abscised were cultured, roots were rarely initiated.

From the two media tested (M&S and WPM), the effect of basic medium on the growth and the survival rate gave similar results as observed for juvenile materials. Axillary shoots grown on WPM medium were dark green and showed good leaf growth, while those grown on M&S medium were pale green and contained small leaves.

The experiment carried out with M&S and WPM media with three levels of sucrose, showed that the effect of sucrose on elongation of axillary shoots, was more pronounced than that of basic medium. However, the beneficial effects of WPM medium for the growth and the survival of explants continued to show.

In a separate experiment the effect of sucrose was tested with 0, 2, 4, 8 and 10% sucrose. As expected, cultures did not survive beyond 8 weeks on sucrose free medium. The mean lengths of the primary axillary shoots increased with the increasing levels of sucrose from 0 to 100 g/l. The quality of the shoots was equally good at both 6% and 8% of sucrose. At 100% the leaves were vitrified, although the shoots had the highest mean elongation. The number of propagules produced increased according to their lengths. Again 6% and 8% produced a similar number of propagules. At 10% sucrose it was a little higher and at 4% it was a little lower. Since the leaves

grown on 10% were vitrified and those on 6% and 8% showed the same result, 6% was chosen to use in later experiments.

In an experiment carried out in the present work, with BAP as the only cytokinin, the mean lengths obtained at both 2 and 16 ppm concentrations were about half of those obtained for 4 and 8 ppm which had similar lengths. The maximum lengths observed at 4 or 8 ppm were about 1 cm even after 16 weeks of culture. High apical dominance was observed in the secondary nodes, showing the growth of only one axillary bud at the top of the node. In the experiment with combinations of kinetin and BAP, the best elongation was obtained from the cultures grown on 7.5 ppm kinetin and 3.75 ppm BAP (S-2 hormones). The best leaf growth too was observed in this medium.

2iP was not as effective as BAP or combinations of BAP and kinetin. However, 2 and 4 ppm showed elongation of about 1 cm, but 8 and 16 ppm had similar lengths as control medium. Cultures grown on 2iP media showed good leaf growth throughout the experiment.

However, the continuation of the procedure using nodal explants with conventional hormones was not very successful due to the same reasons experienced with juvenile material. The growth of the axillary buds after secondary and tertiary nodes, was very slow compared to that of primary cultures. One difference between juvenile and mature origin nodal explants was that the juvenile nodes on control medium had highest elongation, while the maximum elongation of mature nodes was observed in the presence of exogenous hormones.

The effect of thidiazuron was promising with mature origin materials to some extent. But there was no proper axillary shoots on these clusters of buds as observed with juvenile materials. However, these could be proliferated by subculturing them onto thidiazuron containing media.

Since the growth was very slow and uneven, the subculture time too varied from 4-8 weeks. The propagule doubling time as calculated from the figure 37 b was 8-9 weeks.

This situation could not be improved by simple treatments such as transferring onto hormone free media, incorporating GA_3 into culture media or growing them on rooting media containing activated charcoal.

Also, the results with mature nodes indicated that the optimum concentration of thidiazuron used for juvenile material could not be applied directly to mature materials. the use of thidiazuron with mature materials requires further experiments on culture media, growth hormones and culture conditions etc. It is also very important to consider the origin of plant materials. Generally, with most tree species, juvenile origin materials are more easy in in tissue culture systems than mature origin explants.

The proliferation rate of juvenile *Tectona grandis* was much higher than that of the mature origin (Gupta et al,1980). However, increase of rooting ability of mature materials from 10% to 60% has been reported after second subculture which could be an added advantage to tissue culture techniques.

In jackfruit (*Artocarpus heterophyllus*) only seedling origin materials have been successful in micropropagation. There had been no successful *in vivo* vegetative propagation method for jackfruit, either juvenile or mature. A substantial root system has been produced by juvenile jackfruit materials *in vitro* (Rahaman and Blake, 1988 a).

A similar situation was observed in avacado as well. While avacado cuttings do not root *in vivo*, the seedling hybrids have been successful in micropropagation.

Almond cuttings too are very difficult to root *in vivo* in both juvenile and adult stages. A rapid multiplication technique has been developed to produce almond trees with good root systems (Rugini and Vermer, 1982). As for teak, improvement in the rooting percentage has been observed in prolonged subculturing.

Soaking of nodal explants in a hormone solution (BAP 10 ppm and IBA 5 ppm) was the

method used by Carron et al (1985) for mature shoots. They could produce plants, but up to now the success of the work is mostly with juvenile material. Carron et al (1989) reported that micropropagation of mature plants required careful preparation of the mother plants. They have produced plantlets from mature clones, but it seemed they had not been able to proliferate cultures. In the present work, soaking explants showed a higher contamination rate. If there was a single non-sterilized culture in one batch it could cause contamination in the other cultures too.

Rooting of axillary shoots was not studied in detail with mature material of *Hevea*. More than half of the shoot tips transferred onto a solid medium containing 2 ppm IBA, (the best rooting medium found with juvenile materials) produced good roots with cyclic lateral roots. The roots produced *in vitro* on juvenile shoots were very similar to the tap roots produced when seed embryos were cultured. With some species difficulties in rooting and plantlet formation of mature origin material have been reported (Cresswell et al, 1982). Also, there are reports of successful micropropagation systems developed for mature elite trees such as teak, *Eucalyptus* and tamarind etc. (Cresswell and Nitsch, 1975).

As can be seen from the results obtained in the present studies, the growth rate and the response to exogenous hormones of mature materials were very low compared to juvenile materials. This phenomenon was frequently observed with woody perennial trees. Juvenile origin materials are always much more responsive than those originated from mature trees. Whether this mature phase could be removed totally to the juvenile phase by manipulating the medium composition, growth hormones and/or culture environment is still unsolved.

Despite this existing problem of maturation, the possibility of multiplying mature trees via *in vitro* techniques and establishing field trials with micropropagated material has been demonstrated for tree species including *Eucalyptus, Tectona grandis, Sequoia sempervirens, Pinus pinaster* and *Pinus radiata* (Gupta, 1980, Boulay, 1985, Pierik, 1990).

Mature *Eucalyptus* was very difficult to propagate by conventional methods. But, superior *Eucalyptus* trees have been produced from mature trees with a high multiplication rate (Mascarenhas et al, 1981).

Many tropical fruit trees, including naturally polyembryonic species such as *citrus*, mangosteen, mango etc. have been micropropagated successfully while, the achievements with *citrus* are in a very advanced stage (Litz, 1985).

With apple, there are reports on possible rejuvenation with subculture during micropropagation. This has been characterized by the increase in shoot production and in the abifpty of shoots to produce roots (Webster and Jones, 1989, Jones and Hadlow, 1989). The same has been reported for plum by Howard et al (1989).

Also, there are reports on enhanced vegetative vigour observed in *in vitro* propagated plants. Apple trees raised by micropropagules have grown more vigorously than those raised by conventional methods (Howard et al, 1989).

Chapter.5
Experiments on Induction of Somatic Embryos of *Hevea.*

5.1. Results

Induction of somatic embryos from young leaf lobes was the main objective of the work reported here. One of the reason to carry out this work was the success, that has been obtained using this technique in cassava which is in the same family as *Hevea*. Cotyledons, stem pieces and root explants too were also tested in later experiments.

5.1.1. Young Leaf Lobes.

(a). Different Levels of 2,4-D.

Very young leaves, 2-8 mm long, harvested from *in vitro* grown juvenile plants were used in all these experiments.

Solid Murashige and Skoog medium at full strength supplied with 2% sucrose was used with 0, 2, 4 and 8 ppm 2,4-D. Media were prepared in 9 cm Petri dishes.

Leaves were removed from *in vitro* grown cultures using a sterile needle and collected in the water in a Petri dish. They were divided into 3 groups according to their size(2-3 mm, 4-6 mm and 7-8 mm) and then placed on the surface of the media. Both abaxial and adaxial surfaces of the leaves were tried. There were 20 replicates for each treatment.

Half of the cultures was incubated in the dark and, the other half under 12 hour photoperiod. Incubation temperature was $25\pm2^{\circ}C$.

After 2 weeks of culture, leaf expansion was observed in 4-6 mm size and, 7-8 mm size leaves in all the media tested. 2-3 mm size leaves showed a swollen shiny appearance after 2-3 weeks of culture. No callus formation was observed in any of the cultures. There was no difference between the two sides, abaxial and adaxial, and therefore this variable was ignored after 2-3 weeks of culture.

Leaves grown on different levels of 2,4-D media were transferred onto a medium containing lower amount of 2,4-D, at 14, 21, 28, 35 and 42 days of culture. This medium contained 0.01 ppm 2,4-D and 0.1 ppm BAP. The rest of the medium composition was kept unchanged.

Cultures were incubated both in the dark and in the light as before.

Neither callus formation nor organogenesis was observed in any of the cultures. Leaf explants survived up to a culture period of 8-9 weeks and then started to turn brown. No difference was observed between dark and light incubation conditions either.

(b). Combinations of 2,4-D and BAP.

The basic medium used in this experiment was similar to that in the previous experiment(solid M&S with 2% sucrose). Media were prepared in square wells instead of Petri dishes. The combination of 2,4-D and BAP tried were as follows:

BAP (ppm)

2,4-D (ppm)	0	2	4	8	16
0					
2					
4					
8					
16					

2-3 mm size and 4-6 mm size leaves were used in this experiment. Four leaf lobes were placed in each square well and, there were 16 replicates for each medium. Cultures were incubated under a 12 hour photoperiod at 25±2°C.

The results obtained were again negative, similar to those reported for the previous experiment. Swelling of the leaf explants was observed in four combinations of media, containing 2 and 4 ppm 2,4-D and 2 and 4 ppm BAP. After 2 weeks of culture half of the cultures on each medium were transferred onto low 2,4-D containing medium (2,4-D 0.01 ppm and BAP 0.1 ppm), as in the previous experiment. They were transferred onto fresh media every

4 weeks time. The leaf explants grown on the media containing either 2,4-D, or BAP at 16 ppm, turned brown after 8 weeks of culture. Others remained green for up to 12-14 weeks of culture with no signs of callus growth embryogenesis or organogenesis.

This experiment was later repeated, but this time cultures were incubated in the dark. After 15-16 weeks of culture, traces of brown callus was observed on three media, 16 ppm 2,4-D + 16 ppm BAP, 16 ppm 2,4-D + 8 ppm BAP and 8 ppm 2,4-D + 8 ppm BAP. These calli were transferred onto the lower 2,4-D containing medium as before, but no further development was observed in any of the cultures.

(c). Combinations of 2,4-D and Kinetin.

This experiment too, was carried out with the same basic medium and under the same culture conditions as before, but media were prepared in 3.5 cm Petri dishes. The following combinations of 2,4-D and kinetin were used. Cultures were transferred onto fresh media every 4 weeks.

2,4-D (ppm)

kinetin (ppm)	0	2	4	8	16
0					
.5					
1					
2					
4					

Leaf expansion and swelling of cultures were observed after 2-4 weeks of incubation in the light (Plate 16.c). Leaves were turned brown and died after 12-14 weeks, although they were transferred onto organogenesis medium between 14 and 21 days of culture.

(d). Combinations of 2,4-D and 2iP.

This experiment was carried out exactly in the same way as the previous experiment with 2,4-D and kinetin. The hormone combinations used were as follows:

2iP (ppm) \ 2,4-D (ppm)	0	2	4	8	16
0					
2					
4					
8					
16					

Even the results obtained in this experiment was similar to those observed in the previous experiment. Leaf explants showed expansion and swelling, but no further development. Experiment was terminated after 16 weeks of culture.

5.1.2. Cotyledons of Mature Seeds.

For these experiments, cotyledons were excised from mature seeds of clone RRIM 600 and PB 86. The following media were used to culture the cotyledons.

I. 2,4-D at 0, 2, 4, 8 and 16 ppm.

II. Combinations of 2,4-D and BAP as used in for young leaf lobes(page 156).

M&S solid medium supplied with 2% sucrose was used in 9 cm Petri dishes. There were 20 replicates in each medium and the cultures were incubated both in the light and in the dark.

The whole cotyledons and halves were cultured while both abaxial and adaxial sides of them

were cultured.

After a few days of culture, all the cultures except those on 0 ppm 2,4-D medium, incubated in the light, turned green and started to produce callus. Cultures incubated in the dark produced light brown callus, but to a lesser extent (Plate 16.a). Those on 2,4-D free medium did not produce any callus. After 1 week of culture, about half of the cultures were lost due to severe fungal growth, possibly caused by the internally contaminated seeds. After 1, 2 and 3 weeks of culture some calli were transferred onto lower 2,4-D containing medium (0.01 ppm 2,4-D and 0.1 ppm BAP). After 4 weeks of culture, calli on low 2,4-D containing media and on original media started to produce roots. Some calli with roots were transferred onto a medium containing 4 ppm BAP and 0.1 ppm 2,4-D. No organogenesis, except more root formation, was observed in all these cultures.

Even after 16 weeks, only response observed was rhizogenesis in all the media tested.

5.1.3. Roots and Stem Explants.

In this experiment, *in vitro* grown roots and *in vivo* grown stem pieces of juvenile origin were used.

M&S medium supplied with 2% sucrose was used in square wells. Combinations of 2,4-D and BAP was used as used for young leaf lobes (page 156).

For stem culture, young stems of about 5-10 cm were sterilized with $HgCl_2$, as described for shoot tip and nodal explants. They were first cut into 0.5-1 cm long cylinders and then into two halves, horizontally. The outer green skin was removed and the inside woody part was transferred onto the medium, facing the flat side of the explant down.

After 4 weeks of culture, callus formation of *in vivo* grown stem pieces were scored as follows:

0 - no callus
+ - very little callus
++ - some callus
+++ - good callus
++++- very good callus

BAP (ppm)

2,4-D (ppm)	0	2	4	8	16
0	0	0	0	0	0
2	++++	++	++	++	+
4	+++	+++	+++	+++	+
8	++	++	++	+	+
16	++	++	+	+	0

Table 9. Callus formation of stem pieces on media containing BAP and 2,4-D (n=20).

As shown in Table 9, no callus formation was observed on 2,4-D free media. Highest concentrations of either 2,4-D or BAP was not beneficial to produce callus. 2,4-D at 2 ppm showed the best callus growth in absence of BAP. 2,4-D at 4 ppm was also produced good callus when the BAP level was less than 16 ppm.

The calli produced on most of the media were friable, and white or light brown in colour. Callus formation started at the two ends of the stem and then grown all over the explant.Good callus growth could be maintained by subculturing them onto fresh media every 4 weeks.

Some of the calli were transferred onto low 2,4-D containing medium (0,01 ppm 2,4-D and 0.1 ppm BAP), but no organogenesis or embryogenesis was observed in any of the treatments.

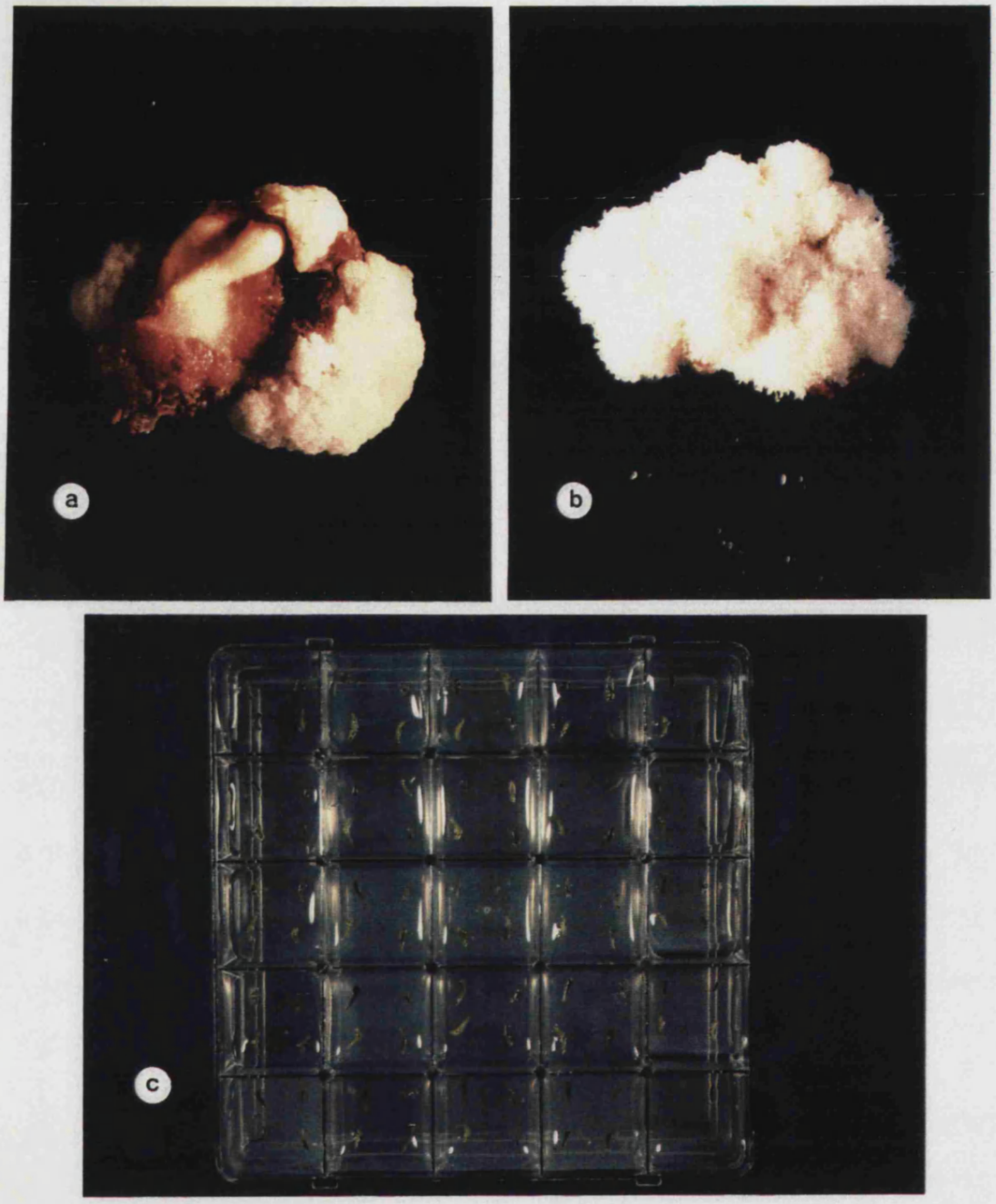


Plate 16 (a). Induction of callus on cotyledon explants at 2 ppm 2,4-D.Results after 8 weeks; **(b).** Callus formation on root explants on medium containing 2,4-D and BAP, each at 2 ppm. Results after 16 weeks; (c). Young leaf lobes, on combinations of 2,4-D and kinetin. Results after 8 weeks.

For root culture, roots were harvested from the *in vitro* cultures grown on hormone free medium and cut into 1 cm size pieces and cultured directly onto media containing 2,4-D and BAP as before.

Results after 4 weeks of culture are shown in Table 10.

2,4-D (ppm) \ BAP (ppm)	0	2	4	8	16
0	0	0	0	0	0
2	+	+++++	++++	++++	0
4	++	++++	+++	++	0
8	++	++++	++	++	0
16	+	+	0	0	0

Table 10. Callus formation of root explants on media containing 2,4-D and BAP (n=20).

As shown in Table 10, 2,4-D was essential for callus formation. But, the increase of 2,4-D from 2 ppm to 16 ppm decreased the amount of callus formation, while 16 ppm 2,4-D with higher levels of BAP prevented callus formation totally. 16 ppm BAP showed no callus formation at any level of 2,4-D. The best callus formation was observed with 2, 4, and 8 ppm 2,4-D (Plate 16.c). As can be expected, the increasing amounts of BAP in the medium decreased the formation of callus.

Good callus of light brown colour, was obtained as explained above, but no further development into embryogenesis or organogenesis of these calli was observed.

5.2. Discussion

Micropropagation of *Hevea* by means of somatic embryogenesis gained the experimental interest of research workers over the past decade, due to the number of advantages it contained over the other tissue culture techniques. Among the various explants tested only anthers and nucellus tissue have given encouraging results so far.

In the present studies attempts were made to induce somatic embryos from young leaf lobes mainly because, this technique has been very successful for cassava (*Manihot esculenta*), which is a member of the family Euphorbiaceae, as *Hevea*.

In cassava, somatic embryos were induced from immature leaves of 15 cassava genotypes (Szabados et al, 1987). Somatic embryos developed directly on the explants on a medium containing 2,4-D at 4 to 16 ppm, although differences were observed with respect to the embryogenic capacity of the explants of different varieties. Plantlets were developed from primary and secondary embryos in the presence of BAP and lower concentrations of 2,4-D. Somatic embryos were also induced from cotyledons and embryonic axes of seeds of cassava in addition to young leaf lobes (Stamp and Henshaw,1982).

In the present work with *Hevea*, immature leaves were removed from *in vitro* grown plants. In the first experiment 2,4-D was tested at 0, 2, 4 and 8 ppm and the only response observed was swelling of leaves which were about 2-3 mm size. Both 4-6 mm and 7-8 mm leaves showed expansion of leaves.

Although the response observed in the first medium was little, the swollen leaf lobes were transferred onto regeneration medium (developed for cassava) after 14, 21, 28, 35 and 42 days of culture. This medium contained 0.1 ppm BAP and 0.01 ppm 2,4-D (personal communication). Neither callus nor organogenesis was observed in this medium and the leaf explants were dead after 8-9 weeks of culture.

The similar results were obtained when combinations of 2,4-D and BAP (0, 2, 4, 8 and 16 ppm of each) were tested. Either 2,4-D or BAP at 16 ppm was too strong and all the explants

were dead by the end of 8 weeks. The rest remained in green up to about 12-14 weeks with no signs of callus growth, embryogenesis or organogenesis.

Cultures grown either on combinations of 2,4-D and kinetin, or on combinations of 2,4-D and 2iP too, behaved in the same way as the combinations of 2,4-D and BAP.

After all these unsuccessful attempts with young leaves of *Hevea*, cotyledons of mature seeds, *in vitro* grown roots and *in vivo* grown stems were tested for their ability to produce embryogenic callus or somatic embryos.

The response of cotyledons to culture medium to produce callus was very high compared to that of young leaf lobes. After a few days of culture, callus growth was initiated on explants on 2,4-D containing media. Media free of 2,4-D showed no callus formation. The resulting calli were transferred onto lower 2,4-D containing medium as before, where they started to produce roots.

Good calli were obtained from stem explants and roots too, in the presence of auxins. Callus growth could be maintained by subculturing the calli onto fresh media every 2-3 months.

However, there was not a single incidence of producing somatic embryos or embryogenic callus, from the thousands of leaf lobes cultured onto many different media.

Carron et al (1984) used fragments of epicotyls, petioles, internodes and cotyledons from which even an 'embryogenic callus' could not be obtained. After several sub cultures, callus growth stopped and roots were formed, as experienced in the present work with cotyledons.

Callus could be obtained from discs of mature leaves in the presence of 2,4-D (results are not reported here). The callus production rate of leaves was very low compared to that of stem pieces, roots or cotyledons.

Induction of roots seems to be fairly easy with most of the explants of *Hevea*. Roots were eventually formed on callus originated from many explants in the presence of auxins or in absence of cytokinins.

The progress, that has been made with somatic embryogenesis of anthers (Wang et al, 1980)

is in an advanced state compared to that of nucellus tissue. The first anther derived plants were produced by Chinese workers in 1977, after about 5-6 years of research work carried out by the scientists around the world (Chen et al, 1977). Since then, the technique has been improved, but still this is a very slow process. Formation of embryos from the callus alone requires 5 to 6 months. This is cultivar dependent and only a few clones have been successful so far. Also flowers must be collected when most of the pollen grains are at the uninuclear stage. This is related to the morphological characteristics which vary with clone and season etc. Moreover, the success rate of plantlet formation is still around 1% which is very low. According to Chen (1984) the success rate of anther culture, though very low, is partly dependent on the incorporation of coconut water into the medium, and partly on the use of sucrose at a level of 7 to 8%. It has been confirmed that the calli formed were entirely somatic origin and that the pollen grains degenerated at a very early stage in the culture. Considerable variation in chromosome number, has been observed in young leaves of pollen derived plants, but the number increased from 9 to 36 during the process of growth (Chen et al, 1990).

After extensive and time consuming research work carried out by the French workers since 1979, plantlets were produced from nucellus tissue via somatic embryogenesis (Micnaux-ferriere and Carron, 1988). It was found that the embryogenic cells on callusing medium continued to develop, only if subculturing was carried out when they were in a specific physiological stage. The optimum period for subculturing obviously depends on the experimental conditions chosen, the type of explants and the clones. True bipolar embryos were obtained by accurate timing of sub cultures and by proper balance of hormones, minerals and sugar in the culture medium. Furthermore, the development stage of the seed from which the nucellus tissue was taken, seemed to be critical. They recommended the seeds after 45 to 75 days of anthesis, to harvest nucellus tissues.

They used four step procedure which takes minimum of 3 weeks for the first callusing medium, 3 to 5 weeks for the second callusing medium, and 5 to 6 months for the differentiation

of embryoids which takes another 4 months for the multiplication of embryoids. This stage was successful with only one clone (RRIM 600) and the differentiation frequency was only 1%. The time required for the final stage of maturation of embryoids and plantlet formation is not yet certain, because the plantlet formation was a very rare occurrence. Carron et al (1989) reported of obtaining only a few tens of plants out of thousands of embryos produced.

As it can be seen the processes of somatic embryogenesis from both explants, anthers and nucellus, consist of more than one critical stages. The media requirements and culture conditions too are far more different for the two explants.

From the experience gained throughout the years of working with *Hevea*, Carron et al (1989) suspected whether the regeneration capacity via somatic embryogenesis in *Hevea*, is located only in the floral organs. One of the reasons to believe this could be the results obtained from the experiments conducted towards this aim with various explants of *Hevea* ; only the anthers and nucellus tissue from seeds were responsive. Another reason could be that in woody species regenerating callus have mostly derived only from embryonic explants; mature and differentiated cells from adult plants generally do not show this ability (Reilly and Washer, 1977; Lakshmi Sita,et al, 1979).

In theobroma cacao, while immature embryos give rise to somatic embryos from their cotyledons, the older embryos, ovules, pericarp or leaf pieces formed only unorganised calli (Pence et al, 1979).

The experiments carried out with leaf explants towards somatic embryogenesis were all discontinued, because on one hand, the results obtained in the present work and by the other workers towards the somatic embryogenesis were not encouraging, and on the other hand, the explants which are expected to be more embryogenic, (anthers, nucellus, embryos, cotyledons, etc.) were not available for the present studies.

However, the difficulty in the use of somatic embryogeneis technique to *Hevea* is supported by the little progress that had been made with anthers and nucellus tissue over about ten years of

time.

Difficulties have been reported in regeneration of woody perennial plants (Bonga, 1982) because of the apparent loss of regeneration potential in mature tissues of woody plants. Although differentiation has been demonstrated from callus derived from cultured embryos and seedlings of several tree species, callus derived from explants of mature origin has lost most of its regenerative potential (Brown and Sommer, 1982). Somatic tissues of flowers of many plants have a high capacity for vegetative reproduction, possibly because of their proximity to the rejuvenating sexual cells (Nozeran, et al, 1971). According to them, a dedifferentiation of cells occur just before or shortly after flower induction. Therefore, explants should probably be taken from flowers at an early development stage. Evidently, floral parts within the mature tree posses a high degree of juvenility, thus high degree of regenerative potential *in vitro*. It has been suggested that this is due to the occurrence and closeness of cells undergoing meiosis which may have a rejuvenating effect on surrounding cells (Litz, 1985).

Chapter.6

Incorporation of 14C-acetate and 14C-Mevalonate into Stem Slices of *Hevea.*

6.1 Preliminary Experiments

The general experimental scheme for the estimation of the incorporation of radioactive precursors into rubber by stem slices was as follows:

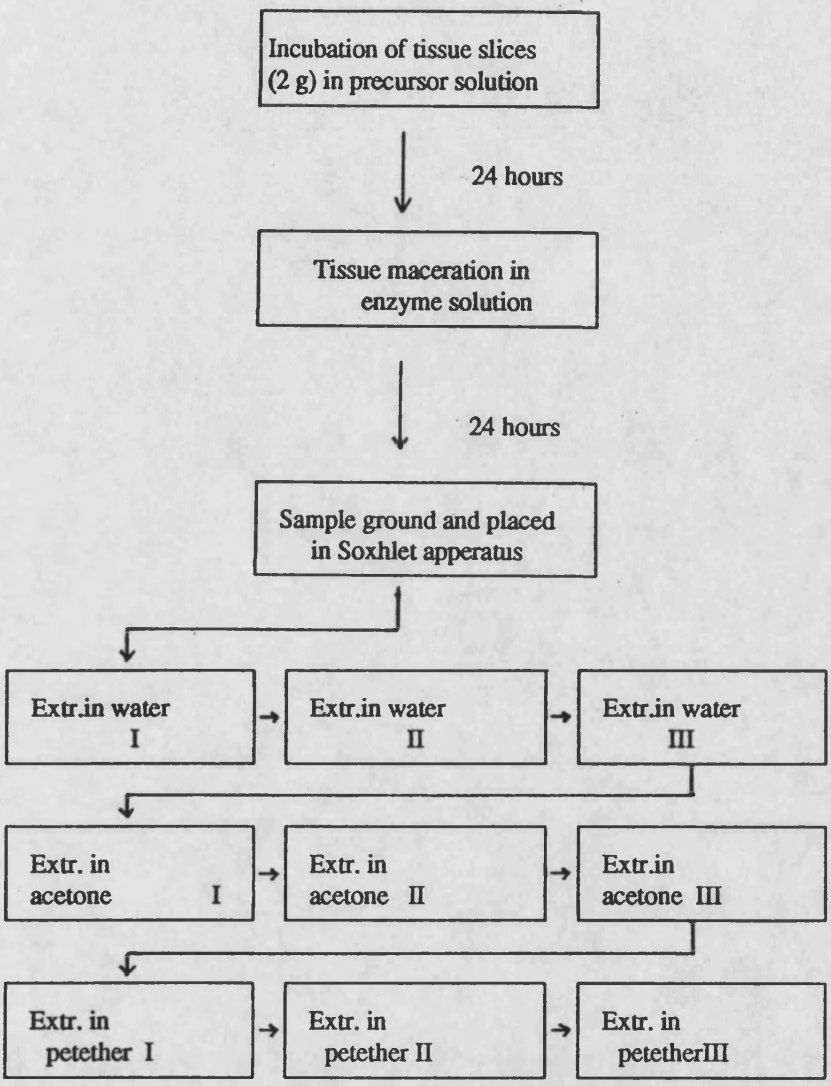


Fig 43. General experimental scheme for the estimation of the incorporation of radioactive precursors into rubber by stem slices.

The uptake of ^{14}C-acetate or ^{14}C-mevalonate by the tissues was calculated by the difference in radioactivity between two samples taken from the preincubation and after incubation media.

After incubation with the precursor (^{14}C acetate or ^{14}C mevalonate), the stem slices needed maceration prior to extraction in the Soxhlet apparatus. A mixture of enzymes (cellulase + pectinase, each at 1%) was very effective for the maceration of young tender stems but, with more mature woody stems, further mechanical maceration was needed.

At first, the incorporation reactions were terminated by washing the samples three times with boiling, 80% ethanol (Macrae, 1986). Later the use of ethanol to terminate the reaction was found to be unimportant. In fact the subsequent enzyme treatment was adversely affected by the ethanol absorbed into the stem slices. Therefore, at the end of the incubation period, the stem slices were washed three times with distilled water to remove any excess ^{14}C-acetate or ^{14}C-mevalonate.

To determine the total final volume of the solvent at sampling,the Soxhlet extraction flasks were initially weighed together with the Soxhlet extractors. In later experiments, however, the final volume was determined by weighing the flasks without the Soxhlets, because it was found that the radioactivity of the solvent in the Soxhlet extractor was only about 1% of that in the soxhlet flask after any extraction period.

6.1.1 The Effect of the Length of Extraction Time-

Soxhlet extraction in all three solvents was carried out for various lengths of time, to determine the minimum time required for near-quantitative extraction of radioactivity from the samples.

Very young shoots of clone RRIC 100 were used with ^{14}C-acetate as the precursor. There were 4 replicates. 1 ml of sample was taken out from the solvent after each extraction period (3x8 hours, 3x3 hours or 3x1 hour). Proprietary cellulose thimbles were used in this experiment for the Soxhlet extraction. Radioactivity recovered was calculated for each sampling with each solvent. Results are shown in Figures 44(a), (b) and (c).See also Appendix 28.

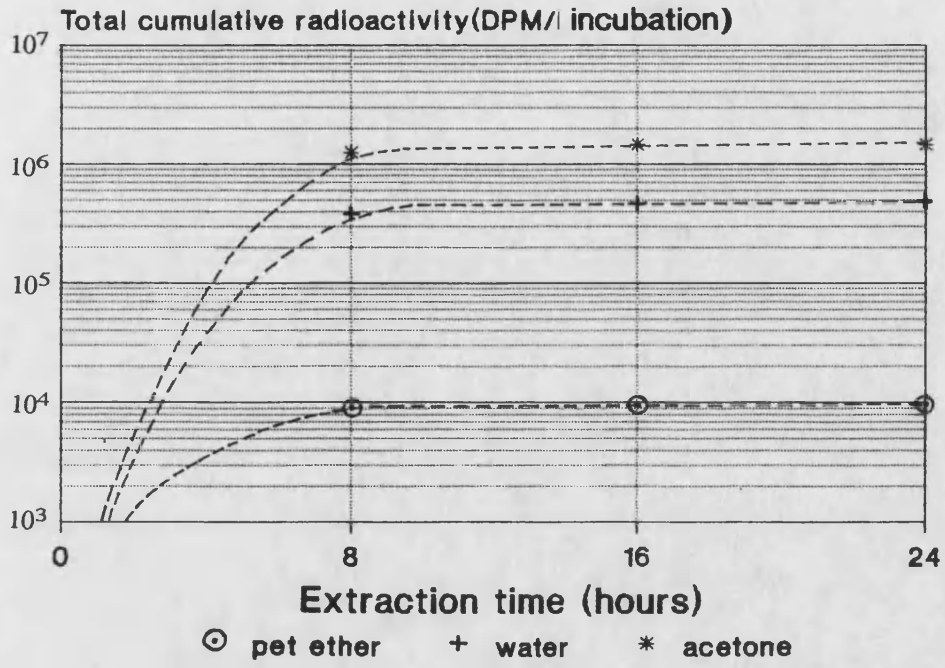


Fig 44(a). Radioactivity recovered in each fraction; three consecutive 8 hour extractions.

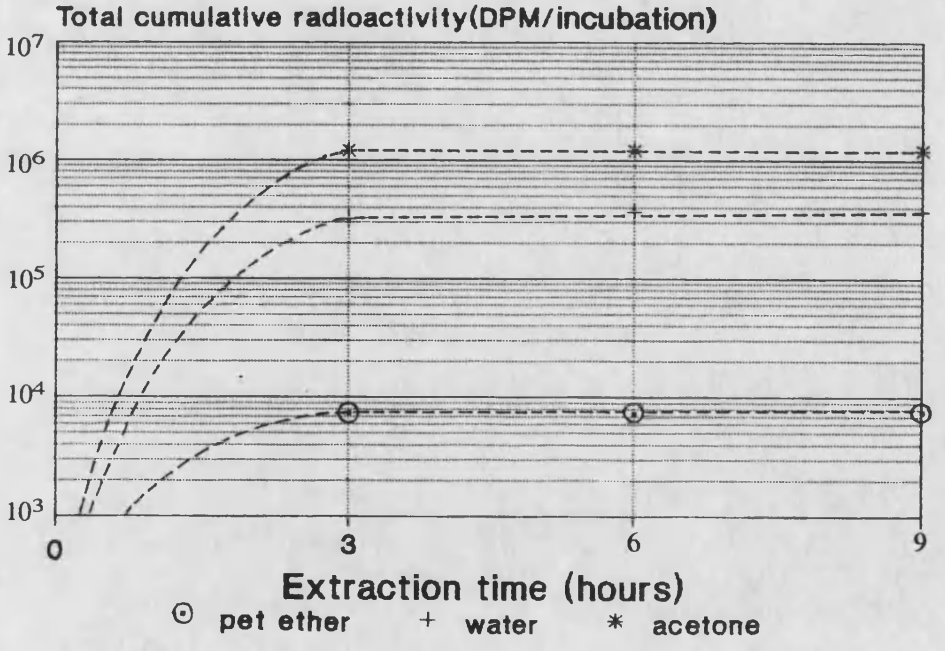


Fig 44(b). Radioactivity recovered in each fraction; three consecutive 3 hour extractions.

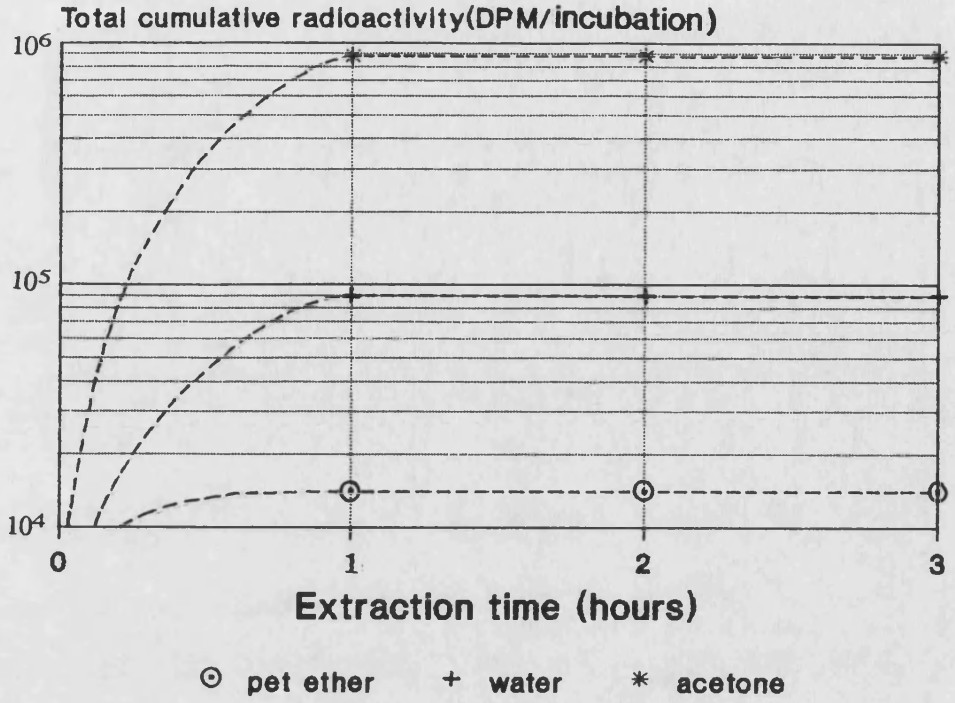


Fig 44(c). Radioactivity recovered in each fraction; three consecutive 1 hour extractions.

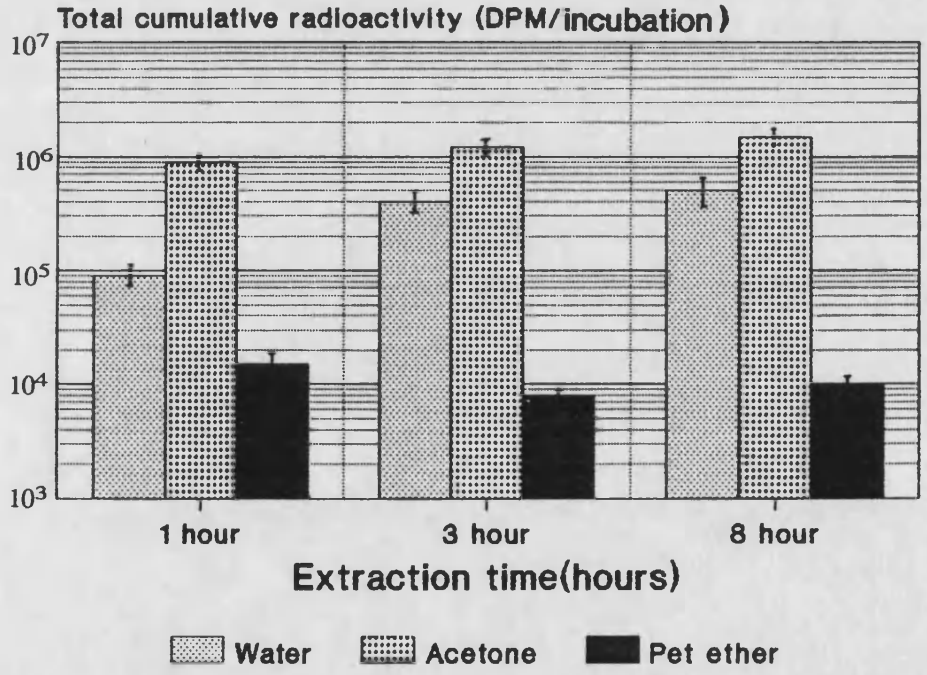


Fig 44(d).Summary of the data in Figures 2(a),(b) and (c). Total radioactivity recovered in 3x8 hours, 3x3 hours and 3x1 hour extractions with water, acetone and petether.

As the Figures show, the total amount of radioactivity recovered in water and acetone has slightly increased with the increasing length of extraction time from 3x1 hour to 3x8 hours. On the other hand the petether extraction was as effective or apparently even more effective after 3x1 hours as after 3x8 hours.

Since the main object of this work was to estimate incorporation of precursor into rubber, and it is the petether solvent which extracts this, it was decided to limit the extraction time to 3x1 hour periods in all experiments that follow.

6.1.2 The Effect of the Type of Thimble on Reproducibility of Results.

Proprietary cellulose Soxhlet extraction thimbles used in the previous experiment cost one pound each; trying to economise on this it was therefore decided to use thimbles made by the author out of Whatman No.1 filter papers instead.

In this experiment very young shoots of clone RRIC 121 were incubated with ^{14}C-acetate.Three consecutive one hour extractions were carried out with each solvent.

Results of an experiment replicated three times was carried out with hand made and proprietary thimbles are given in Figures 45 (a), (b), (c) and 46 (a), (b) and (c);see also Appendix 29.

Figure 45 shows that the standard error of the mean was very large at every sampling time for all three solvents when using hand made thimbles for the extractions. On the other hand when proprietary thimbles were used, the standard errors of the means were very small (Fig 46). Moreover, the hand made thimbles interfered with the proper siphoning action of the Soxhlet apparatus, by blocking the side arms; this resulted in increasing the length of time required for near-quantitative extraction. Furthermore, the hand made thimbles were not strong enough and in some instances they disintigrated while in use.

It was therefore decided to use only proprietary thimbles in all future experiments.

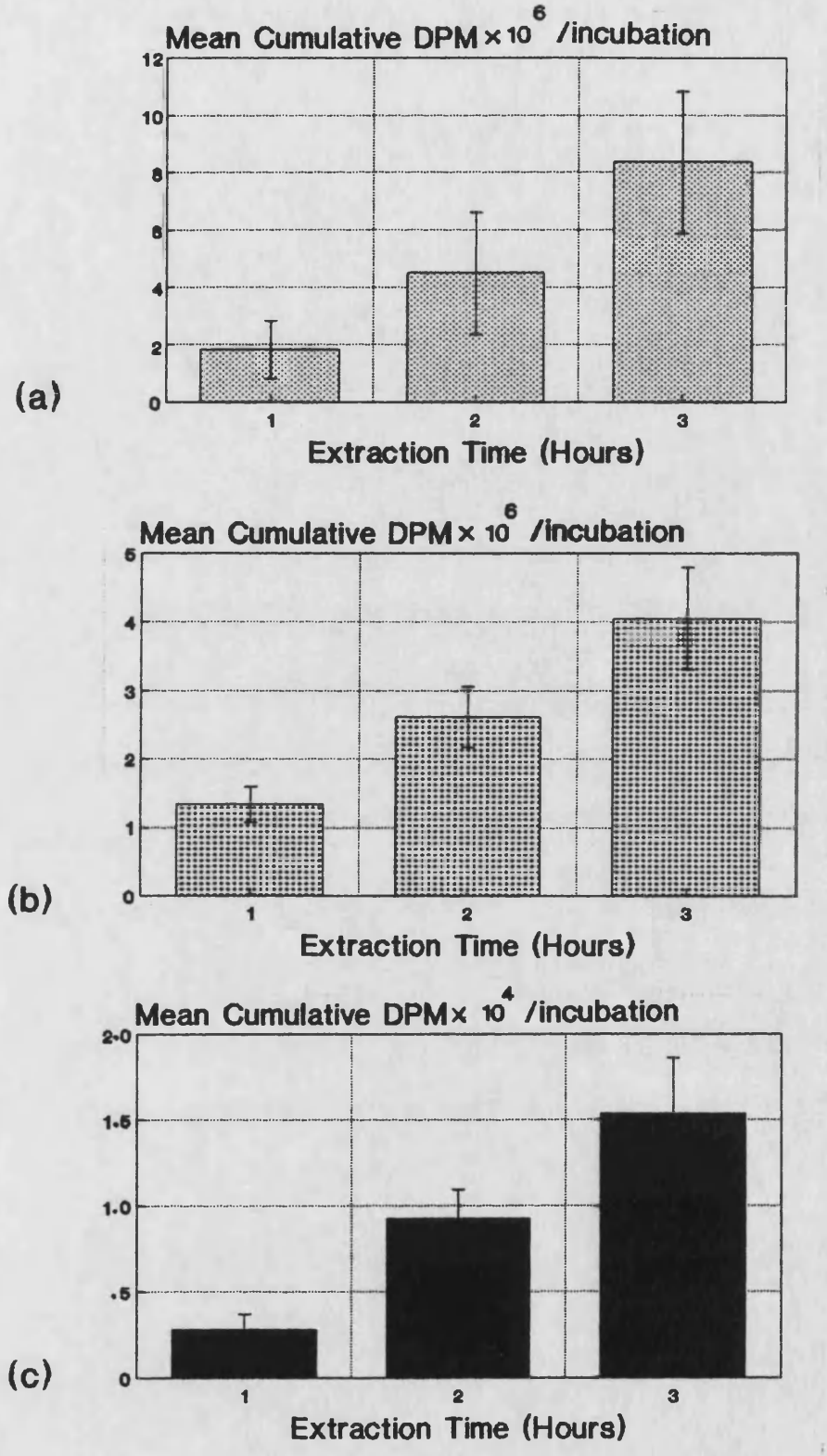


Fig 45. Radioactivity recovered in water(a), acetone(b) and petether(c) in experiments carried out with hand made thimbles (n=9). Error bars show SEM.

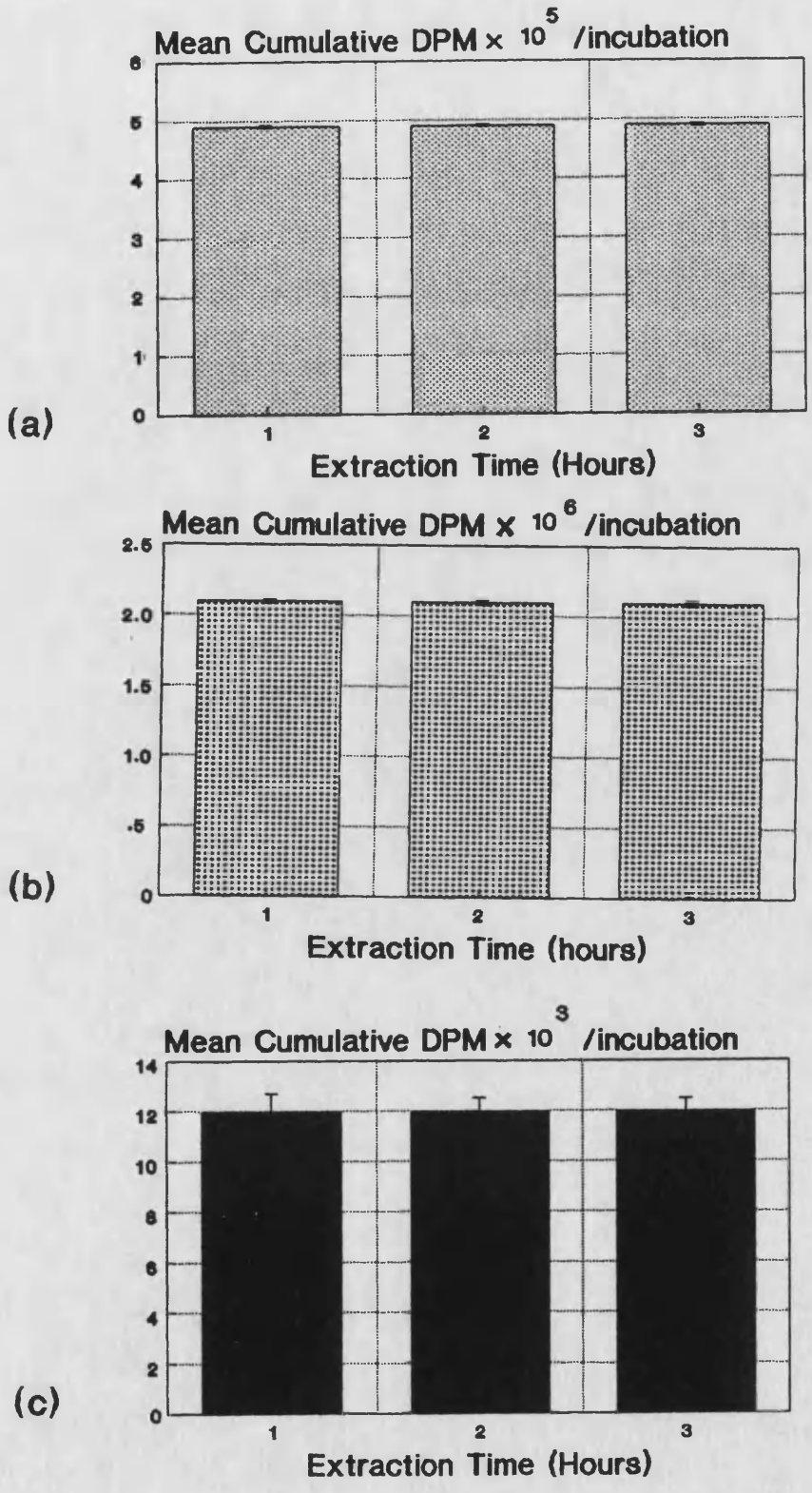


Fig 46. Radioactivity recovered in water(a), acetone(b) and petether(c) in an experiment carried out with proprietary thimbles (n=3). Bars show SEM.

6.2 Results

The work reported in this chapter is based on the results of incorporation of rubber precursors into rubber by the stem slices of *Hevea.*

Only ^{14}C-acetate and ^{14}C-mevalonate were used as rubber precursors in the present studies. The technique as used for guayule by Macrae, et at, 1986 was used with minor modifications, to determine the rubber producing potential of the experimental material. Plant material was available from five clones of which the the long term yield potential was known from work done at the Rubber Research Institute of Sri Lanka.

In order to develop the *in vitro* assay method, variuos potential sources of variation in the incorporation rate such as the nature of the precursor, daily variation of the plants, physiological state of the plant material, within clone variation and between clone variation were studied.

6.2.1 Comparison of ^{14}C-acetate and ^{14}C-mevalonate as precursors.

As a first priority, it was decided to look at the extent of uptake and incorporation of two different precursors into rubber by stem tissues. The precursors used were ^{14}C-acetate and ^{14}C-mevalonate.

The specific activity of ^{14}C-acetate and ^{14}C-mevalonate used were 2.04 and 2.11 MBq/um respectively. The radioactivity of the incubation medium for both precursors was adjusted to be in the range from $3.0x10^6$ to $1.1x10^7$ DPM and therefore the carrier concentration of the precursors in the incubation medium was in the range of $2.5x10^{-6}$M to $8.3x10^{-6}$M. The pH of the medium was not buffered, but was measured to be 5.6 ± 0.3.

The uptake of precursor was presented as a percentage of the total initial radioactivity in the incubation medium. The mean uptake into the tissues of the two precursors is shown in Figure 47.The results are based on 117 incubations with acetate and 39 incubations with mevalonate.

As shown in Figure 47, the uptake of the two precursors were significantly different; the acetate uptake was more than twice that of mevalonate.

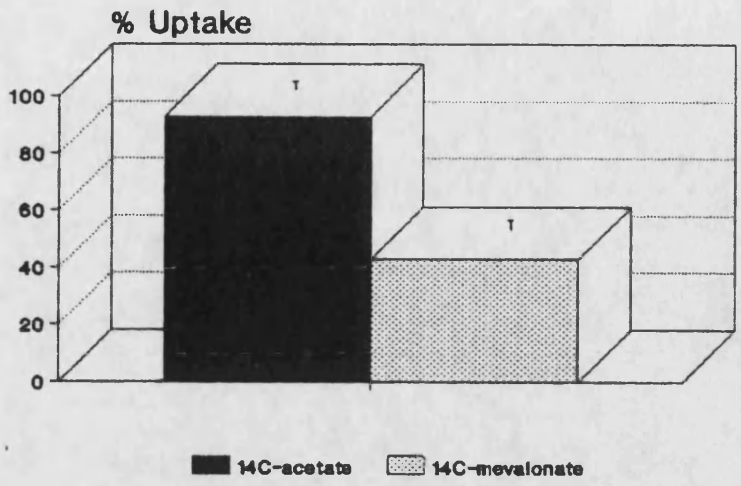


Fig 47. Uptake of ^{14}C-acetate and ^{14}C-mevalonate by stem slices (for acetate n=117, for mevalonate n=39).Bars show SEM.

When the amount of radioactivity incorporated into the petether fraction was expressed as a percentage of uptake, the difference between the results obtained using the two precursors is much smaller (Fig 48). The results shown in Figure 48 were from an experiment carried out with two clones, RRIC 110 and RRIC 121. There were three replicates for each treatment but only 2-3 plants were used from each clone (see also Appendix 30).

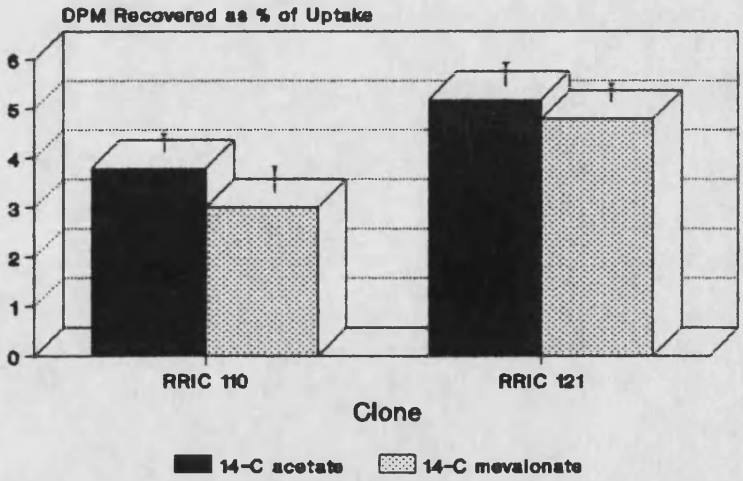


Fig 48. Comparison of incorporation of ^{14}C-acetate and ^{14}C-mevalonate into rubber by stem slices of clones RRIC 110 and RRIC 121 (n=3). Bars show SEM.

Figure 48 shows that the rubber incorporation was not significantly different for the two precursors, although the ^{14}C-acetate incorporation was slightly higher in both clones.

6.2.2 Daily Variation of ^{14}C-acetate Incorporation.

To study this, a single plant was used from clone RRIC 121. Mature shoots, as defined in the section 6.2.3, were removed on 3 successive days. Stem slices (fresh weight approximately 2g per incubation) were incubated with ^{14}C-acetate. Nominally, the initial concentrations of ^{14}C-acetate in all incubations were identical but, in fact, they varied somewhat as actually measured.

The relationship between initial acetate concentration and uptake is shown in Figure 49(a), see also Appendix 31.

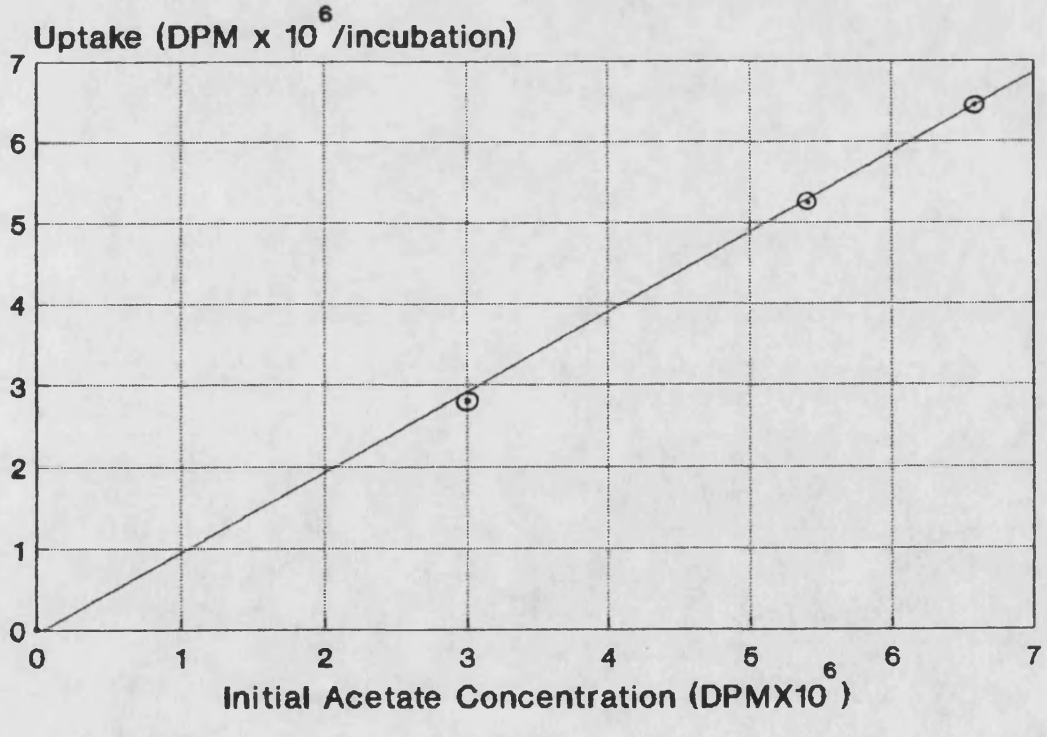


Fig 49(a). The relationship between initial ^{14}C-acetate concentration and uptake (n=3).

As may have been expected, at such low concentrations the uptake increased with initial concentration.

The incorporation of acetate into rubber for the three successive days expressed in terms of percentage of uptake is shown in Figure 49(b), see also Appendix 32.

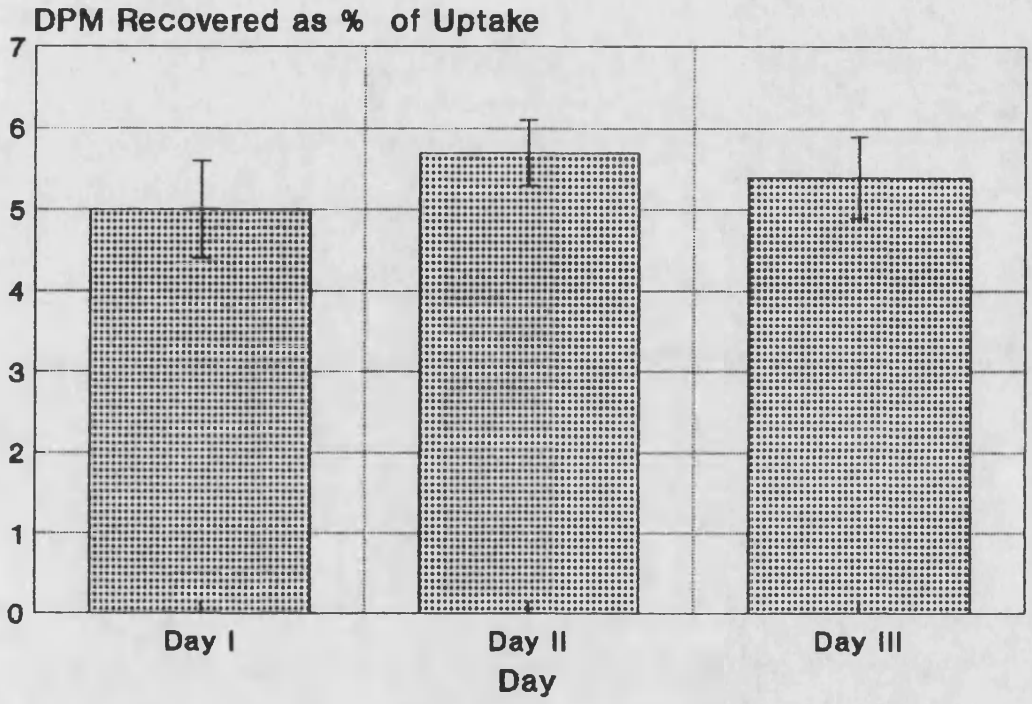


Fig 49(b). Daily variation of the incorporation of ^{14}C-acetate into rubber by stem slices of the same plant (n=3). Bars show SEM.

It can be seen from the Figure 49b, that the acetate incorporation percentage does not vary significantly between the three experiments carried out on three different days, even though the uptake varied between 92 and 98% of the initial acetate applied, and of course there is an inevitable variation between incubations carried out on the same day.

6.2.3 The effect of the Physiological State of the Experimental tissues on 14C-acetate incorporation.

The growth of *Hevea*, like that of many other tropical woody species, occurs in flushes (Figure 50). The total duration of a growth cycle is about 12 weeks which can be subdivided arbitrarily and approximately into three stages. During the first few weeks, shoots elongate. This is followed by a period lasting about three weeks, in which the leaves mature. Finally, a stationary phase of approximately another five weeks follows, completing the growth cycle.

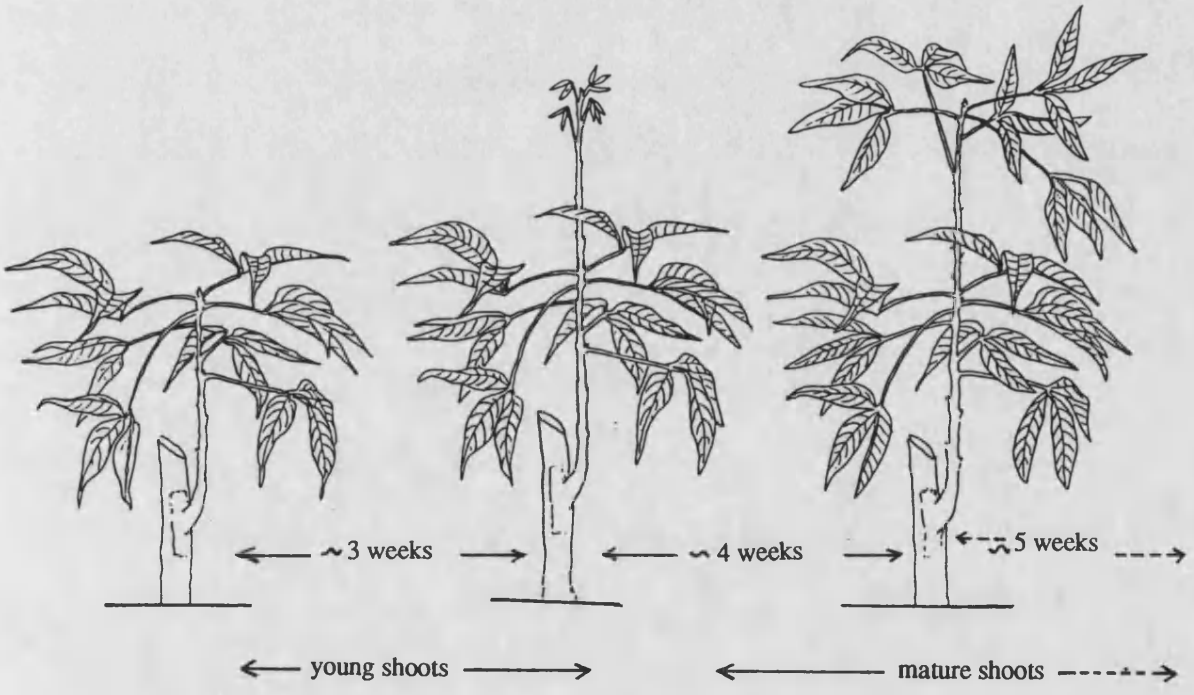


Fig 50. Normal growth pattern of a clonal *Hevea* tree in the glasshouse.

In the first, actively growing phase, the stem is tender and green and the young unexpanded leaves are copper brown. This type of shoot is described as a 'young shoot' in the present work. In the third, stationary phase, the stem becomes woody although it is still green. The shoots in this stationary state of growth were called 'mature shoots'.

Two experiments were carried out to investigate the effect of the physiological state of the

experimental tissues on ^{14}C-acetate incorporation.

In the first experiment, two types of shoots, young and mature were used. Stem pieces were removed from the same branch of a single tree of clone RRIC 117.

Stem slices (total fresh weight approximately 2g) from each type of shoot were incubated with ^{14}C-acetate. The young stem slices were macerated adequately by the enzyme treatment, but the slices cut from mature stems needed additional mechanical maceration. Each treatment was replicated three times. Radioactivity recovered in the petether fraction was expressed as a percentage of uptake.

The mean values obtained for the incorporation of ^{14}C-acetate into rubber as a percentage of uptake for the 'young' and 'mature' shoots were 2.1+0.4% and 3.5+0.4% respectively (see also Appendix 33). The means show that the incorporation percentage for mature shoots was almost twice as high as for young ones.

Since the percentage incorporation is likely to be related to the dry matter content of the experimental material, and since the dry matter content of mature shoots was found to be significantly higher than that of young shoots (see section below), these results were not unexpected. The difference between the two means was found to be significant at 6.6% level using a one sided t-test. The use of this version of the t-test rather than that of the usual two sided version, was warranted here (Rowntree, 1981) because of a prior likelihood of the mean dry weight for the mature shoots being the higher of the two. The relatively low level of significance is no doubt related to the small number of replicates.

The dry weights of the two types of stems were also measured. 2g of stem slices of each type, removed from the same tree, were dried in an oven at 40 C for a week, until constant weights were obtained. Three replicates were used and mean dry weights were evaluated. The dry weights were 21+0.05% of the fresh weight for the mature shoots and 10+0.05% of the fresh weight for the young shoots.

Since the results indicated that the higher incorporation of ^{14}C-acetate by mature stem slices

could be related to the higher content of dry matter of the same compared to the young materials, a second experiment was carried out.

In this experiment, only mature stem slices were used. Two different weights of stem slices, 1g and 3g were compared for the incorporation of ^{14}C-acetate into rubber. Each sample was weighed from thoroughly mixed stem slices removed from a single plant of clone RRIC 121. Stem slices were incubated with 10ml of ^{14}C-acetate. There were three replicates for each variable.

Radioactivities recovered in the petether fraction expressed as percentages of uptake for the two weights of stem slices are given in Figure 51.

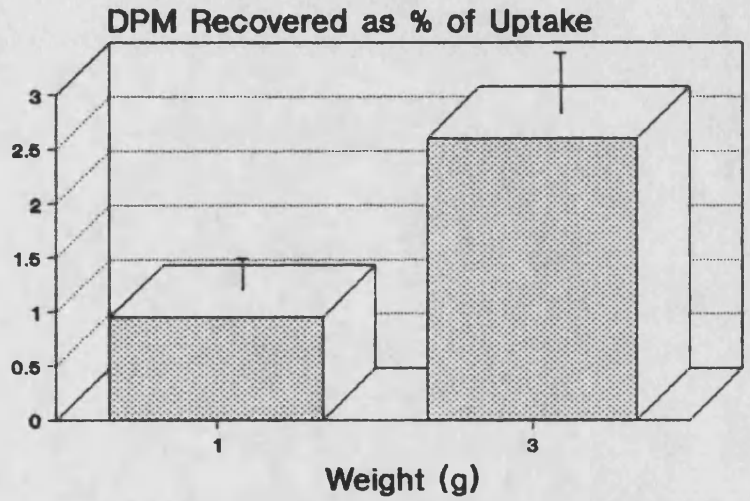


Fig 51. Comparison of incorporation of ^{14}C-acetate into rubber by two different weights of stem slices (1g and 3g).Bars show SEM (n=3).

As Figure 51 shows, that the incorporation of ^{14}C-acetate by 3g of stem slices was 2.7 times higher than that observed for 1g of stem slices.

The mean uptake of ^{14}C-acetate was 96% for 1g of stem slices and 98.8% for 3g samples; although the dry matter content of the two samples varied nearly threefold, the uptake of radioactive precursor was nearly constant.

6.2.4 Variation of ^{14}C-acetate Incorporation among individual trees within one Clone.

Eight individuals from clone RRIC 100 were tested for their potential to incorporate ^{14}C-acetate into rubber. Mature shoots only were used. Only two plants could be tested on any one day. Three replicates were used from each plant. Radioactivity recovered in the petether fraction as a percentage of uptake was evaluated for individual plants. Results obtained are given in Figure 52a (see also Appendix 34).

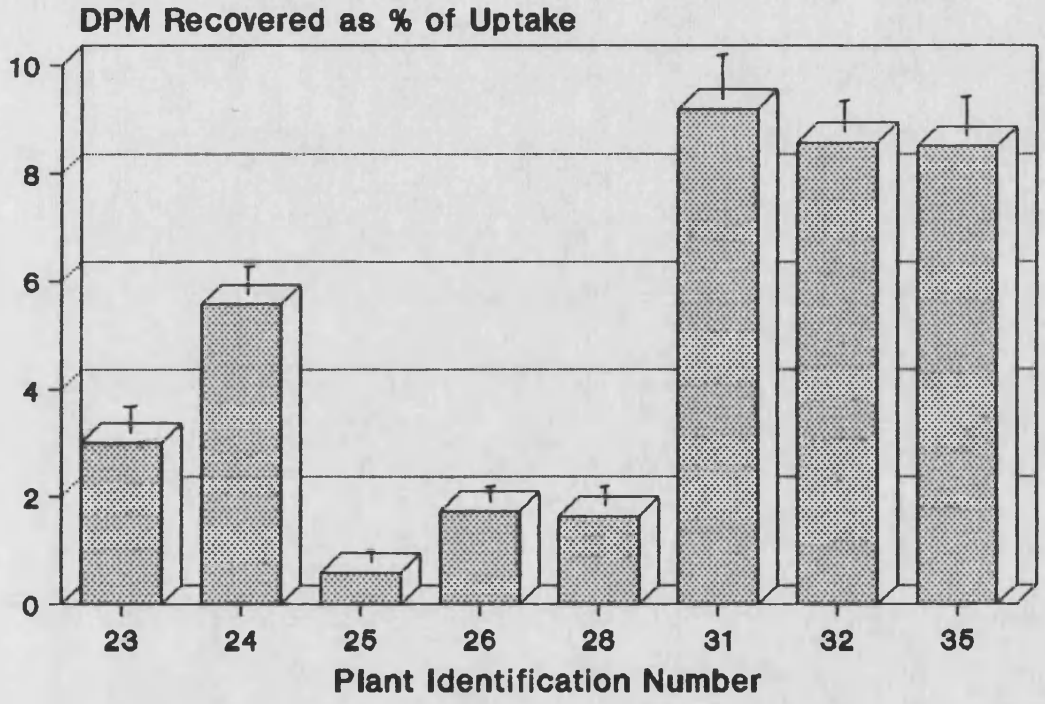


Fig 52(a). Variation in the ability to incorporate ^{14}C-acetate into rubber among individuals of clone RRIC 100. Bars show SEM.

As Figure 52 shows, the variation between individuals in clone RRIC 100 was very high and statistically significant. Variation of this kind could be expected to occur within all clones as a result of the scion-rootstock interaction. The percentage recovery in the petether fraction varied from 0.6% to 9.2%, the mean value was 4.5∓1.4%. This result emphasises the necessity for representative sampling in comparing clones for their rubber producing potential.

To see whether this variation could be reduced by proper sampling, another experiment was carried out in which the incorporation of ^{14}C-acetate into rubber was studied using the same 8 plants of clone RRIC 100 as used in the previous experiment, but with the stem slices randomized before taking samples for incubation. About 4cm long mature shoots were removed from each plant, and slices were mixed well. 2g fresh weight of plant material was then weighed from the mixture of slices. Three replicates were used. Radioactivity recovered in the petether fraction as a percentage of uptake was calculated. The mean figure obtained was 4.76±0.6%. The means between the two experiments (4.5 and 4.76) differ by approximately 6%, while the SEMs (1.4 and 0.6) differ by 80%.

The dry matter content of the same individual plants are shown in Figure 52(b), see also Appendix 34(b).

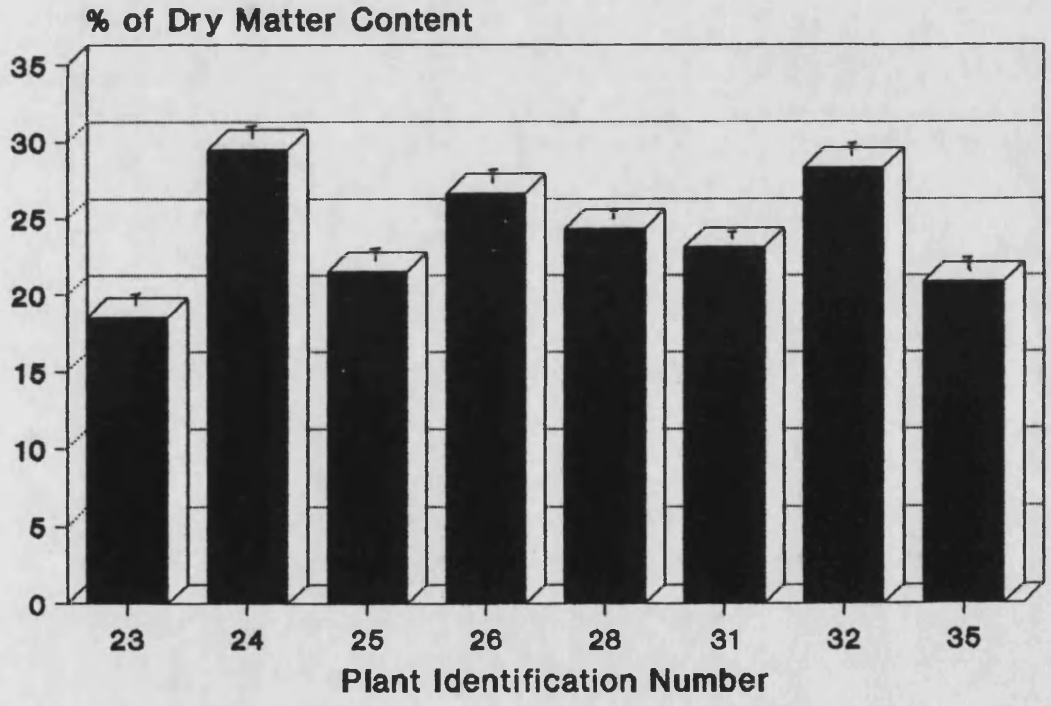


Fig 52(b). Percentage dry matter content of 8 individual plants of clone RRIC 100. Bars show SEM,(n=3).

As can be seen from Figures 52 (a) and (b), there is no direct relationship between the incorporation of ^{14}C-acetate into rubber and the dry matter content of them. But in both cases, high variation among individual plants can be observed.

6.2.5 Comparison of Clones.

In this study, clones PB 86, RRIC 100, RRIC 110, RRIC 117 and RRIC 121 were used. The precursor used was ^{14}C-acetate. Clones were represented by eight to ten plants and samples were taken in triplicate from each clone after thorough mixing of the slices from the same clone. All the shoots used were of mature origin and therefore mechanical maceration was needed after enzyme treatment.

Radioactivities recovered in the petether fractions were expressed as percentages of uptake for each clone. Results are given in Figure 53 (see also Appendix 35).

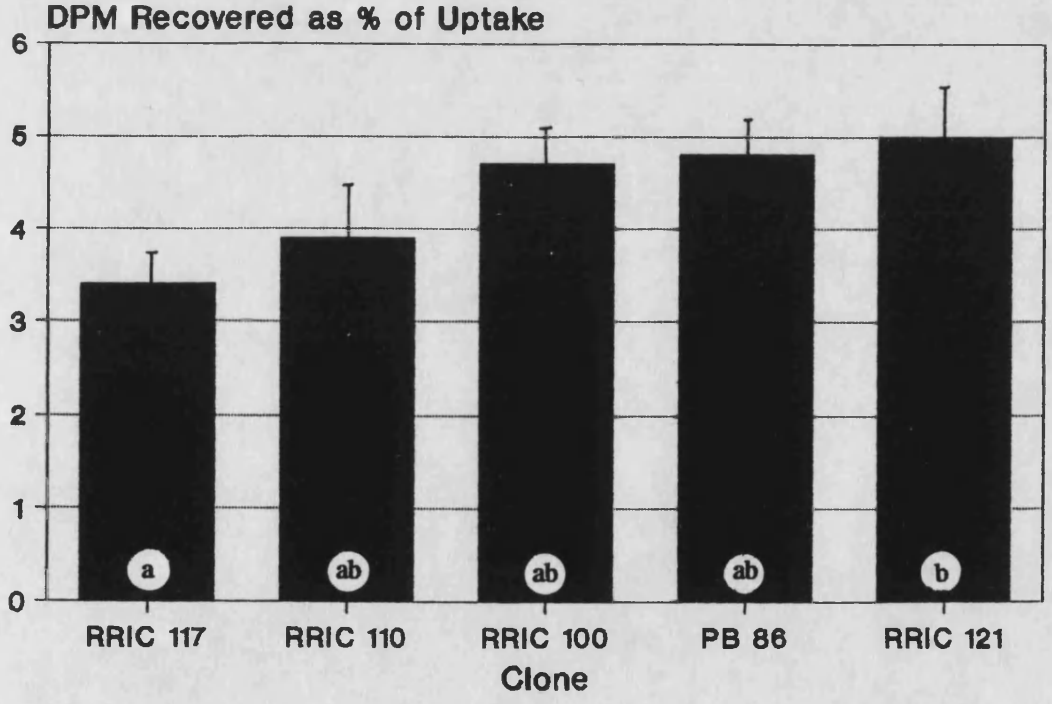


Fig 53. Rubber producing potential of the five experimental clones. Bars show SEM.

As can be seen from Figure 53, the incorporation of ^{14}C-acetate by stem slices varied between clones although the differences were not very large.

Radioactivities recovered as percentage of uptake were 3.4, 3.9, 4.7, 4.8 and 5% for the clones RRIC 117, RRIC 110, RRIC 100, PB 86 and RRIC 121 respectively.

The mean dry rubber yield (Kg/Ha/year) of clones PB 86, RRIC 100, RRIC 110, RRIC 117 and RRIC 121 obtained from field experiments from the Rubber Research Institute of Sri Lanka are given in Figure 54 (see also Appendix 36).

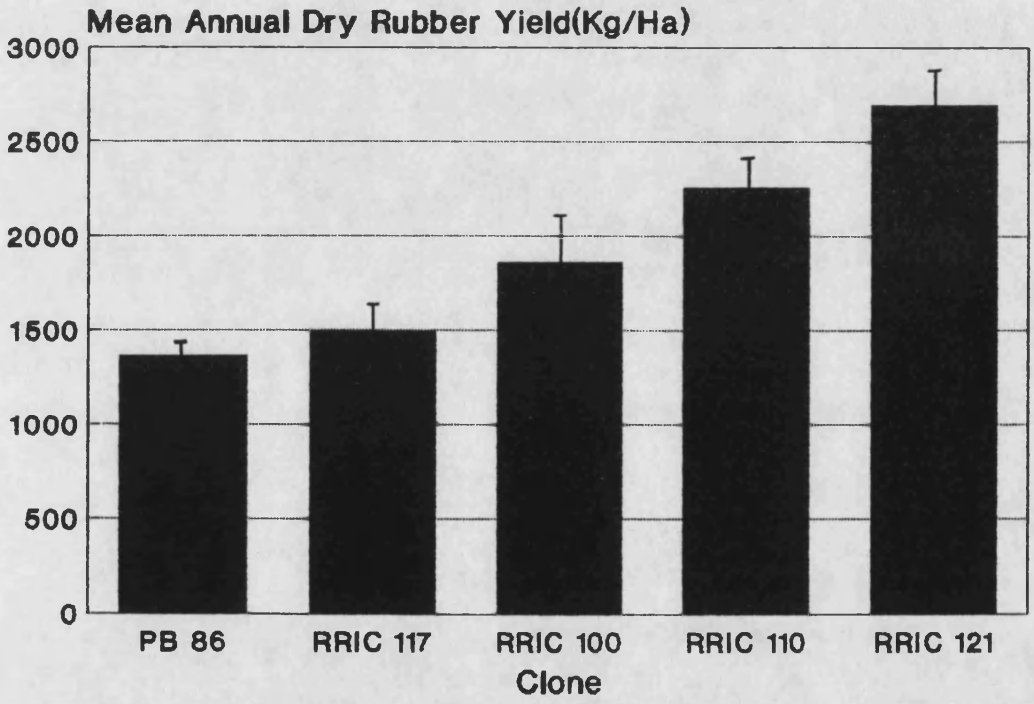


Fig 54. Mean dry rubber yields (Kg/Ha/year) of clones PB 86, RRIC 100, RRIC 110, RRIC 117 and RRIC 121 obtained from field experiments from the Rubber Research Institute of Sri Lanka.

As can be seen from Figure 53, only the clones RRIC 121 and RRIC 117, are significantly different. The other three clones lie in between these two, showing no significant difference between each other.

The sequence of the clones, when arranged in descending order according to their ability to incorporate ^{14}C-acetate into rubber, is as follows;

RRIC 121 > PB 86 > RRIC 100 > RRIC 110 > RRIC 117

According to the mean annual yields (Figure 54), the sequence of clones in descending order is as follows;

RRIC 121 > RRIC 110 > RRIC 100 > RRIC 117 > PB 86

The *in vitro* incorporation percentage sequence and the *in vivo* yield sequence are seen to be homologous to some extent, although the possition of clone PB 86 is clearly anomalous.

6.3 Discussion

The estimation of the ability of tissues to incorporate precursors into rubber by extraction of the petether soluble fraction followed by liquid scintillation counting is a generally accepted procedure.

Gilliland et al (1985) confirmed the presence of ^{14}C labelled rubber in petether extracts by NMR analysis. After the removal of waxes and other impurities from the petether extracts, 92% of the radioactivity recoverable by a single petether extraction was still present in the petether extract. This component gave a NMR spectrum which indicated that it contained over 90% cis-polyisoprene.

In the present studies, the method used by Gilliland et al (1985) and Macrae et al (1986) was used to determine the incorporation of ^{14}C labelled acetate into rubber.

Gilliland et al (1985) used this technique to measure the incorporation of ^{14}C-CO_2 by different parts of guayule (*Parthenium argentatum*) into rubber. The same method was used by Macrae et al(1986) to determine the incorporation of ^{14}C-acetate, ^{14}C-acetyl-Co-A, ^{14}C-mevalonate and ^{14}C-IPP into rubber by stem slices of guayule.

After the incubation period with ^{14}C labelled precursor, Macrae et al(1986) washed the stem slices with 80% boiling ethanol, prior to drying and grinding in a mill. Gilliland et al did not use ethanol because they used ^{14}C-CO_2 a gaseous precursor. The plant material was dried at 60 ^{o}C for 24 hours and ground to a homogenous powder before extraction in the Soxhlet apparatus. In the present work, stem slices were washed only with cold water after the incubation period, since the ethanol was suspected to affect the enzyme treatment which was used for maceration. When mature shoots were used, further mechanical maceration was needed in addition to the enzyme treatment. This was carried out in the incubation flask itself.

An attempt to use thimbles hand-made from Whatman filter papers instead of proprietary cellulose thimbles was a failure as described in the results (Section 6.1.2).

The samples placed in cellulose thimbles were extracted in succession with water, acetone

and petether (40-60°C boiling range). Gilliland et al (1985) extracted samples for 8 hours in each solvent. Macrae et al (1986) tested 8, 16 and 24 hours and found that 8 hours was sufficient. In the present studies 1, 3 and 8 hours were tested, and one hour was found to be sufficient to extract as efficiently as eight hours (Section 6.1.1), and therefore the samples were extracted only for one hour in each solvent.

After the extraction, constant volumes of 10 ml were obtained by Macrae et al (1986) by evaporating the solvent. 1 ml samples were then used to count the radioactivity. Gilliland et al (1985) evaporated the solvent to dryness and then made them up to constant volumes of 50 ml; 1 ml samples were used for counting radioactivity. In the present work, plant materials were extracted with 50 ml of solvent, and 1 ml samples were taken after each extraction period. The total final volume was determined by weighing the solvent in the flask.

Macrae et al(1986) used as many as four rubber precursors in their work with guayule stem slices. In the present studies only ^{14}C-acetate and ^{14}C-mevalonate were used. As reported by Macrae, the incorporation of acetate and acetyl-Co-A was more efficient than that of the other two.

As explained in Section 6.2.1, the uptake of acetate was found to be twice as high as that of mevalonate, 93% and 45% respectively. With stem pieces of guayule (Macrae et al,1986) the incorporation of acetate and mevalonate into rubber were about 16% and 1.6% respectively, when the radioactivity of the petroleum ether fraction was expressed as a percentage of the total radioactivity recovered in the extractions. In the present studies the incorporation was always presented as a percentage of uptake. The previous two workers did not report uptake figures, therefore no direct comparisons with the present work can be made.

There are a large number of reports in the literature dealing with incorporation of precursors into rubber by *Hevea*. In all of these studies, latex rather than tissue slices have been used. The results vary greatly. Bandurski and Teas (1956) demonstrated the incorporation of ^{14}C-acetate into rubber by *Hevea* latex. They also found, that the incorporation of isopentenyl pyrophosphate

(IPP) was considerably lower than that of acetate. This was supported by Harris and Kekwick (1961).

However, rather higher incorporation of IPP was reported by Archer et al (1961 and 1963). Park and Bonner (1958) and Gascoigne and Jones (1959) were unable to obtain incorporation of ^{14}C-acetate under conditions used by Bandurski and Teas (1956).

Park and Bonner (1958), Kekwick et al,(1959) and Archer et al (1963) obtained incorporation of 2-^{14}C-mevalonate into rubber. Again, neither Gascoigne and Jones (1959) nor Kekwick et al (1959) were able to observe incorporation mevalonate into rubber.

The incorporation of acetate by *Hevea* latex was low as reported by most workers, and that of pyruvate was even lower. It seems that the source of the latex has an important bearing on the results obtained by measuring the incorporation of precursors into rubber.

Among the possible explanations given by Archer and Audley for this situation with *Hevea* latex, the following are of importance.

(a).The relatively low population of mitochondria in the tapped latex compared to *in vivo* conditions, implying a reduced generation of acetyl-Co-A by the pyruvate oxidase system.

(b).Production of cyanide from endogenous precursors on incubation, which inhibits *in vitro* respiration. Related to this, the consumption of oxygen by latex is very low.

In the current work the daily variation in the incorporation of ^{14}C-acetate into rubber (Section 6.2.2), was found not to be significant between measurements carried out on three successive days.

This finding was somewhat similar to the results obtained by Woo and Edwin (1970), in an experiment carried out with *Hevea* latex. Latex samples were collected on three days during a 5 day period and the figures obtained for three trees were as in the following table.

According to them the variation between trees and days was not significant but, the variation between the two factors combined was significant.

Tree Number	Mean % incorporation		
	Day I	Day II	Day III
1	11.9	12.8	13.5
2	10.9	5.5	19.5
3	6.9	7.2	5.0

(From Woo and Edwin,1970)

Black et al (1982) reported measurements of the resin and rubber content of guayule, analysed in triplicate on three different days. The resin content of these samples ranged from about 4 to 8%, whereas the rubber content varied from 6 to 18%.

According to Black et al, plant sample nonhomogeneity is probably the single most important variable encountered, which could lead to poor accuracy and reproducibility in the analyses of rubber in guayule.

Rather high standard errors were also observed in the current experiments with *Hevea* stem slices. The possible explanation for this variation could be the same as for guayule reported by Black et al.

In the same experiment, the effect of the initial concentration of ^{14}C-acetate on the uptake was also evaluated. Three concentrations were involved in this experiment, from 2.76×10^{-6} to 5.28×10^{-6} M.

The relationship between the initial concentration and the uptake was linear (Fig 48a) indicating the concentrations used were not too high.

As explained in the Section 6.2.3, the growth of *Hevea* occurs in flushes, and different morphological states can be identified, which probably relate to different physiological states.

In this experiment, stem slices were compared only in two very distinct physiological states,

'young' and 'mature', as described in the Results. Although the same fresh weights were used from both types, the incorporation by mature stem slices was almost twice as high as by 'young' ones. This can no doubt be explained, at least partially, by the dry matter content of the two types of shoots; the percentage dry weights were 21% and 10% for the 'mature' and 'young' shoots respectively.

Present findings with *Hevea* stem slices are in agreement with those of Macrae et al (1988) with guayule stem slices. In guayule, the difference between the incorporation of ^{14}C-acetate by 'young' and 'mature' stems slices was not significant but the incorporation of ^{14}C-acetate by the 'bark' was three times higher than that of other younger parts of the shoot.

Further, Gilliland et al(1985) reported better incorporation of $^{14}C\text{-}CO_2$ by mature stems than by very young shoots tips. The stems collected from ground level showed lower incorporation than the middle part of the stem, but still higher than the top part of the shoot.

In the current work, the results obtained for 'young' and 'mature' shoots were further tested by another experiment, but this time two different fresh weights were compared taken from the same type of plant material. Only mature shoots were used in this experiment. The mean incorporation of ^{14}C-acetate by 3 g of stem slices (fresh weight) was 2.7 times higher than that obtained by 1 g of stem slices. The total initial radioactivity and the volume of the incubation medium were the same for both 1g and 3g. The uptakes were 96% and 98.8% for 1g and 3g of plant materials respectively.

An experiment of this type was also carried out with *Hevea* latex by Archer et al(1963), to study the effect of the rubber content of latex on the incorporation of IPP by latex into rubber. For this, latex samples were prepared by centrifuging fresh latex followed by diluting with buffer, so as to give a series of samples with rubber content ranging from 0 to 23% by weight.

The rate of incorporation of IPP into rubber varied directly with rubber content, up to a concentration of about 15% of rubber.

As reported by Leong et al (1982) the rubber content as well as the dry matter content, varied

with the clone, maturity of the shoots, and the cultural practice. In addition dry weights of different parts of the plant also varied with the clone.

In the present experiment with *Hevea* stem slices to investigate the variation among individual trees within one clone, the recovery in the petroleum ether fraction varied from 0.6 to 9.2%, with a mean value of 4.5+1.4. The dry matter content of the stems of the 8 individual plants used in the previous experiment was also measured. This too, varied among individuals, but it was not the main factor responsible for the variation in the percentage incorporation between individuals within the same clone.

Although this type of variation was not unexpected, the variation was very high in the incorporation of ^{14}C-acetate. Differences in the plant materials such as the possible interaction of the stock and the scion, and thereby the anatomical and physiological differences between the plants could account for the variation.

Variation in vigour, growth pattern and the latex yield etc. among individual trees within any one clone is always observed *in vivo*, in the field. The main reason for this is, in addition to inevitable environmental variation, the poor vegetative propagation method available for rubber; that is, grafting buds from selected clones onto seedling rootstocks. This high variation between individuals emphasises the necessity for representative sampling, when comparing clones for their rubber producing potential or yield.

One of the main objectives of the present work was to see whether the rubber producing potential by stem slices would correlate with their *in vivo* latex yield. If such correlation could be established then the technique could be used as an alternative to select trees for their yield. This would be a very important tool to plant breeders since the conventional method is both time and labour consuming. The findings of the present studies are based on the theory that ^{14}C found in petether was poly isoprene. But, it is possible for ^{14}C precursors to metabolize into other compounds which are also extractable in petether. However, Gilliland et al (19859 have found that over 90% of petether extractable ^{14}C was poly isoprene. In the present studies, given more

time, the accuracy would have been checked by NMR analysis.

If the five clones used in this study are arranged in descending order of yield in the field, as measured by the Rubber Research Institute of Sri Lanka, (see also Figure 53 on page 185), the following sequence results:

RRIC 121 > RRIC 110 > RRIC 100 > RRIC 117 > PB 86

The sequence of the clones according to their ability to incorporate ^{14}C-acetate into rubber, as obtained in the present studies was as follows, although the percentages were not significantly different.

RRIC 121 > PB 86 > RRIC 100 > RRIC 110 > RRIC 117

Although the two rankings are not identical, some relationship between the two can be recognized. Clone RRIC 121 gave the highest latex yield in the field, and also showed the highest incorporation of ^{14}C-acetate into rubber. Further, clone RRIC 117, which showed the least incorporation of ^{14}C-acetate, is one of the two lowest yielders in the field.

As shown in Figure 54 on page 186, the latex yield of clone RRIC 117 is about half of that of RRIC 121. All the others lie in between these two, showing no significant difference between each other.

A somewhat similar picture was obtained in measurements of the incorporation of ^{14}C-acetate in to rubber by stem slices taken from the same five clones (Figure 53 on page 185). But the clone PB 86, one of the two lowest yielders in the field, showed the second highest incorporation of ^{14}C-acetate in to rubber.

Again, the possible explanation for this could be the very high individual variation observed within any one clone. As stated earlier, this emphasises the requirement of a reasonable number of individuals to represent a clone. But in the present studies, only 8-10 plants were available from each clone, while the data of field experiments are based on hundreds of plants. The latex yields of the five clones were mean annual yields; the annual yields varied to some extent with the tapping year as shown below in Figure 54 (see also Appendix 37).

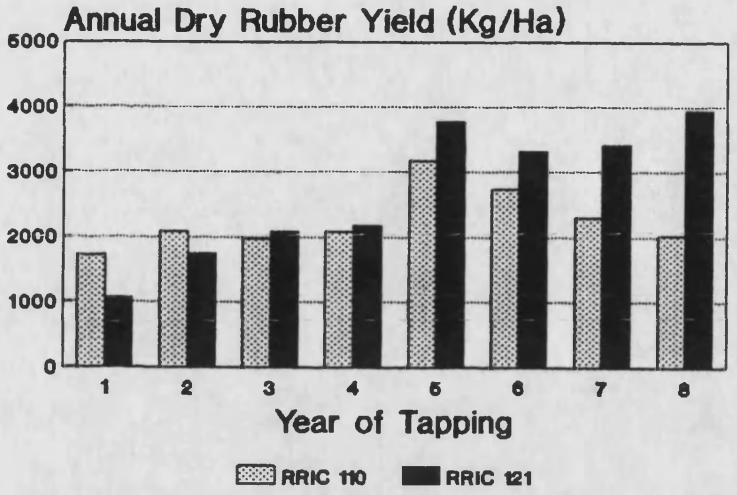


Fig 55. The annual yields of clones RRIC 110 and RRIC 121. Data obtained in field experiments by the the Rubber Research Institute of Sri Lanka.

Whether the rubber producing potential determined *in vitro*, as in the present study varies with the age of the plant tissues used for the measurements, is not known. However, the results obtained so far are encouraging because the plants used in the present work were only two years old, and it was still possible to identify at least two clones, with extreme latex yields. The insufficient number of replicates used must also have affected the results, because of the very high individual variation.

Woo and Edwin (1970) also tried work out some relationship between the latex yield of *Hevea* clones and and rubber biosynthesis *in vitro* using *Hevea* latex. Fresh latex from *Hevea* trees was incubated with radioactive precursors and the efficiency of rubber biosynthesis was measured. The annual yields of the clones tested had some relationship with the incorporation of precursors but they could distinguish only those clones the yields of which were either very high or very low. They have also pointed out the fact that the individual variation within each clone was large compared with the variation between clones.

Chapter.7
General Discussion

As far as micropropagation of *Hevea* by node culture is concerned, one striking feature observed was the difference between plant materials of 'juvenile' and 'adult' derivation. Since *Hevea* is a woody perennial, this was not surprising or unexpected; as with many other woody perennials, juvenile and mature materials of *Hevea* too, have several characteristics, unique to their physiological state of growth.

In the present studies, two types of juvenile materials were used; the plantlets obtained *in vitro* by embryo culture and shoots derived from rootstocks. Embryo cultured plants were only a few weeks old when the nodes were harvested, but the rootstock plants were about 2-3 years old when they produced the shoots used as explants.

In culture, no difference was observed between the nodes of these two types of juvenile material, although the ages of them were different. It seems that an age difference of 2-3 years is not as important as is their origin, because both types had originated from seeds.

When a juvenile tree reaches a certain age, it becomes mature. The time required for this maturation varies with the species. In *Hevea*, flowering occurs, when the trees are about 4-5 years old; this is true for both seed-derived and bud-grafted trees.

The mature (clonal) materials used originated from five known clones. When the shoots were harvested, the clonal plants were about 1-2 years old, since having been grafted but, since they were propagated through bud grafting, they are automatically in the adult stage (Kester, 1976) and eventually showed mature characteristics.

The first difference observed between the juvenile and adult material was, the difficulties in culture establishment. Mature materials were more difficult to establish in culture due to high percentage contamination and severe phenolic browning. This was overcome by sterilizing shoots with $HgCl_2$, together with PVP in the medium to reduce browning.

In culture, the main difference between the two types was the slower growth rate and less responsiveness to culture media / growth hormones observed with mature materials.

The initiation of buds visible to the naked eye has been possible when using adult materials

from most tree species but to trigger elongation of such buds has proved difficult (Boulay, 1987). Because of this problem, successful protocols for the micropropagation of adult trees are rare. Nevertheless, the multiplication of mature, selected trees, using axillary buds has succeeded with some angiosperm trees, even without pretreating the plant material (Bouley, 1987). Successive transfers on multiplication medium improved the rooting ability of the *in vitro* produced shoots. However, the *in vitro* propagation of mature trees has not been achieved for most tree species and the problem of rejuvenation still remains.

The mechanism of rejuvenation is not yet clear. It appears that cells in an undifferentiated state help rejuvenation because the cells in meiosis are in an undifferentiated state (Chen and Evans,1990). Many important questions, such as how the old information is removed from the cytoplasm, how the cytoplasm gets new information, and when the nuclear genetic information is reprogrammed, are still unanswered. (Chen and Evans, 1990).

The present work showed promising results with juvenile materials. With mature materials bud proliferation was observed in the presence of thidiazuron but, shoot elongation was not observed in any of the cases. Rejuvenation of mature materials must be worth investigating. But, such studies were not carried out in the course of this work because both plant materials and time were limiting.

Among the several methods, that have been used for other species to rejuvenate mature materials, repeated grafting of mature buds onto seedling roots, either *in vivo* (Fig 2 on page 7) or *in vitro* (micrografting), would be worth trying for *Hevea*, because, bud grafting is a common as well as an successful technique used for rubber.

Muzik and Cruzado (1985) were successful to some extent in inducing juvenile characteristics in 8-10 year old rubber trees by repeated bud grafting. The extent of juvenility was measured by the ability to produce roots on the resulting plants. In their experiments, rooting was observed on the cuttings taken after 4 repeated graftings. The mature trees used by them were only 8-10 years old; whether these originated from seeds or from grafted buds, has not been

reported. The existing commercial clones of *Hevea* have been maintained for over many years; it is therefore very difficult to determine the real age of grafted clonal plants.

When using conventional hormones to induce axillary shoot growth of juvenile materials, the growth became slower and weaker with each culture passage. It was found that, if juvenile axillary shoots were allowed to root prior to taking nodes, the elongation of axillary shoots of these nodes was significantly better than that of those taken from non-rooted cultures (Section 3.1.7b). The disadvantage of this method is the extra time required; the rooting passage required another 4-8 weeks, which slowed down the proliferation rate. Since mature materials showed no continuous proliferation at all, even a very slow proliferation would be useful, if continuous.

Roots, being the major supplier of cytokinins in plants, may be providing the buds with a particular combination of hormones in correct amounts, which is not easy to supply exogenously.

In this combined method, a rooting passage follows each shoot induction passage and, all the nodes are from rooted plants (Fig 56). In the present studies only a limited version of this method was put into practice. Only one passage of rooting was tried with secondary and tertiary nodes, and this showed promising results with juvenile materials with a mixture of kinetin and BAP (no thidiazuron).

In later experiments, juvenile nodes cultured with thidiazuron, showed far better results than with conventional hormones. Therefore the combined method had no advantage for use with juvenile materials; but it might serve as a rejuvenating method for mature materials. However, no attempts have so far been reported to rejuvenate mature materials of *Hevea* by any other workers. Only the low responsiveness of mature materials in tissue culture systems has been mentioned (Carron et al, 1989).

As for many other species, somatic embryogenesis would be the best solution for mature materials of *Hevea*, since it would guarantee true rejuvenation while propagating. As discussed in Chapter 5, this has not been easy with *Hevea*, even by using the best explants available. There is enough evidence that cells and tissues associated with sexual reproduction in older trees, such

as nucellus tissue , ovules or somatic anther cells may be induced to undergo embryogenesis more easily than other parts of the same plants. In agreement with this, in *Hevea* too, only the anthers and the nucellus tissue have responded in producing somatic embryos so far. However, even for these two explants the growth stage, composition of media, subculture period etc. seem to be very critical. In both cases somatic embryos were produced through embryogenic callus, and the success rate was very low and limited.

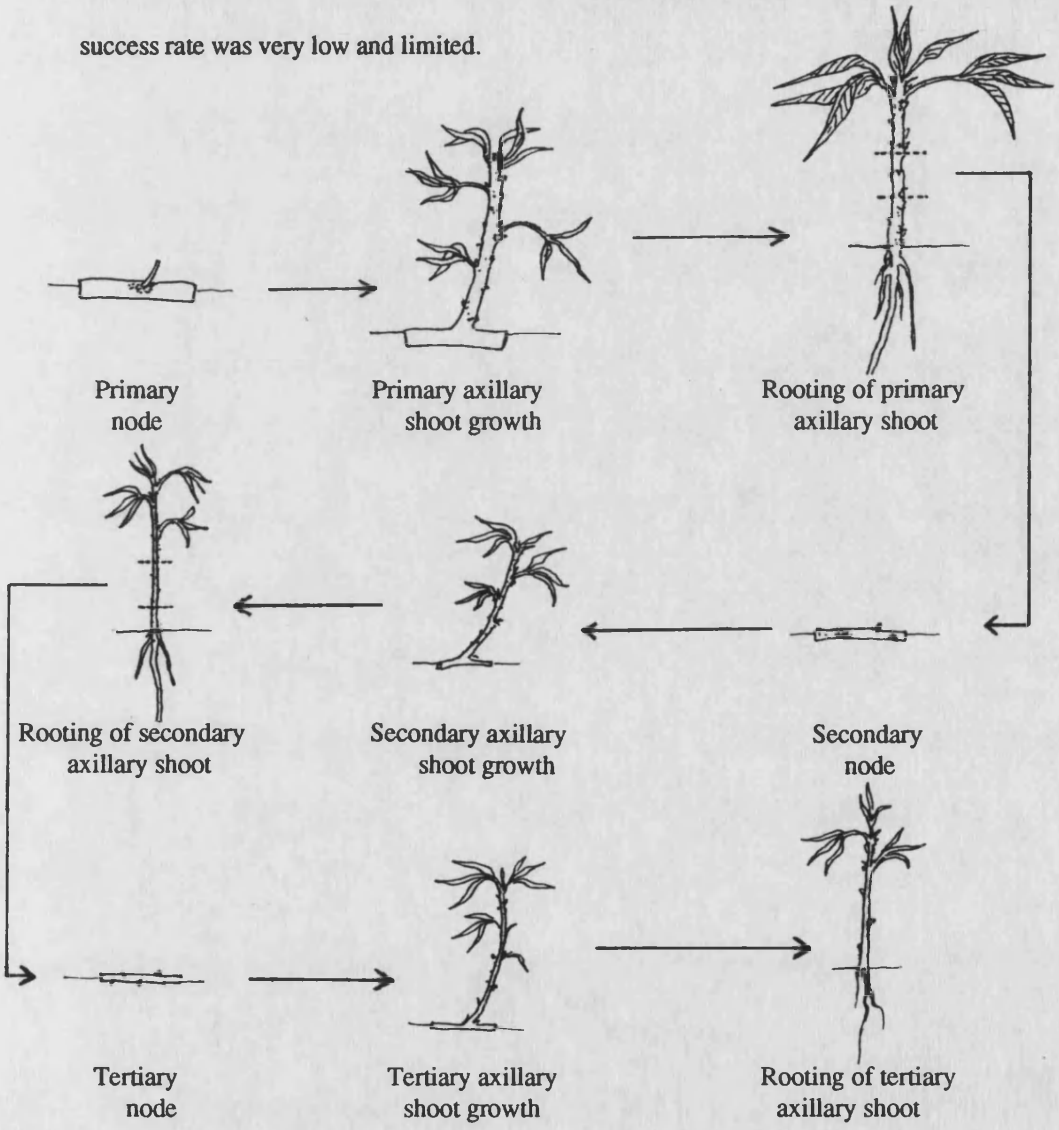


Fig 56. Use of rooting as a tool to induce axillary shoot growth of resulting nodal explants.

Generally, the rooting of cuttings in *Hevea* under *in vivo* conditions is not easy. Even under a mist spray, only a fibrous root system is produced. However, rooting was rather easy under *in*

vitro conditions. The roots produced on most of the shoots were tap roots with cyclic lateral roots (Plate 10), as in embryo-cultured plants. Mature shoots, too, produced good roots on the medium developed for juvenile shoots, but at a lower rate. Experiments were not carried out to optimize the rooting of mature shoots because shoot proliferation was of the first priority.

Thidiazuron may be effective in producing multiple axillary shoots from mature materials, but it seems that such materials need rejuvenating first.

As far as the incorporation of radioactive labelled precursors into rubber is concerned, the results obtained were somewhat encouraging. The main objective of the work was, to see whether a correlation existed between the incorporation potential of the experimental clones and their known rubber yields *in vivo*.

If such correlation could be established then the technique could be used to select individual plants, possibly from micropropagation, at an early stage. This would be a very useful tool for plant breeders, because the conventional method of selecting clones is based on their latex yield. A tree can be tapped when it is about 5 years old. The latex yield varies to a considerable extent with the age of the plant for another 5-6 years (Fig 55 on page 195). Therefore, a reliable alternative method for selection could be very useful. The main shortcoming of the present experiments was the limitation of plant material and of time. Although a large variation among individuals within a clone was observed, only 8-10 plants were available to represent an entire clone. However, the clones at the two extremes of field yield, could be identified in the present work. Since the precursor incorporation technique requires only a small piece of stem, the latex yield of individual trees in the field, could be directly compared with the results of this technique.

Latex of *Hevea* has already been used by Woo and Edwin (1970), towards the same objective as in the present work. One advantage of a method using stem slices instead of latex is that it can be applied to plants at a very young age before they start to produce latex on a large scale. Also the use of stem slices was easier and is possibly more reliable, as the stem slices are closer to the natural conditions of the plants than is latex.

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Appendices

Appendix 1. Mineral and Organic Constitution of Microcuttig Medium (Carron et al,1989).

Macronutrients (mg/l)	
NH_4NO_3	825
$CaCl_2.2H_2O$	988
KNO_3	228
$MgSO_4.7H_2O$	740
$NaH_2PO_4.H_2O$	1360
Micronutrients(mg/l)	
H_3BO_3	9.3
$MnSO_4.H_2O$	16.9
$ZnSO_4.7H_2O$	11.5
$CuSO_4.5H_2O$	.4
$CoCl_2.6H_2O$	.2
$Na_2MoO_4.2H_2O$	.2
KI	.8
Na_2SO_4	92.3
$FeSO_4.7H_2O$	27.8
Na_2EDTA	37.3
Organic ingradients(mg/l)	
Thiamine	10.0
Glutamine	192.0
Sucrose(g/l)	60.0

Appendix 2. Composition of Woody Plant Medium (Lloyd and McCown,1980).

	mg/l
Majour nutrients	
NH_4NO_3	400
$CaCl_2.2H_2O$	96
$MgSO_4.7H_2O$	370
KH_2PO_4	170
K_2S0_4	990
$Ca(NO_3)_2.4H_2O$	556
Micro nutrients	
H_3BO_3	6.2
$MnSO_4.H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.25
Na_2EDTA	37.3
$FeSO_4.7H_2O$	27.8
Vitamins and Additives	
Inositol	100
Nicotinic acid	.5
Pyridoxine.HCl	.5
Thiamine.HCl	1.0
Glycine	2.0

Appendix 3. Plant Growth Regulators and their Solvents.

Growth Regulator	Mol.Weight	Solvent
Cytokinins		
BAP	225	KOH/NaOH
Kinetin	215	KOH/NaOH
Thidiazuron	220	DMSO
Auxins		
2,4-D	221	EtOH
NAA	186	KOH/EtOH
IAA	175	KOH/EtOH
IBA	203	KOH/EtOH
Gibberillines		
GA3	346.3	EtOH

Appendix 4(a). Mean lengths of axillary shoots produced by root stock derived nodes and shoot tips (n=8).

	Mean length of axillary shoots (mm)				
Time(weeks)	4	8	12	16	20
Nodes	4	5.9	5.9	7.3	7.3
Shoot tips	1	1.25	2	2	2.2

Appendix 4(b). Mean number of axillary shoots per explant of root stock derived nodes and shoot tips (n=8).

	Mean number of axillary shoots per explant						
Time(weeks)	4	8	12	16	20	24	28
Nodes	1	1.25	1.38	1.5	1.5	4.0	4.0
Shoot tips	0.66	0.77	0.77	0.77	0.77	1.38	1.38

Appendix 5(a). Mean lengths of axillary shoots of nodal explants produced on four media (n=13).

Time (weeks)	Mean length of axillary shoots (mm)			
	S-0	S-1	S-2	S-3
4	10.4	6.4	4.9	4.6
8	12.3	9.9	5.6	5.1
12	18.3	10.5	5.8	5.4

Appendix 5(b). Shoot proliferation of nodal explants on four media (n=13).

Time in weeks	N_O				N_t			
	S-0	S-1	S-2	S-3	S-0	S-1	S-2	S-3
0	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1
8	1	1	1	1	1	1	1	1
12	1	1	1	1	1.3	1.3	1.7	1.5
16	1.3	1.3	1.7	1.5	4.9	3.2	2.9	1.9
20	4.9	3.2	2.9	1.9	4.9	3.2	2.9	1.9
24	4.9	3.2	2.9	1.9	5.5	4.4	3.5	1.9
28	5.5	4.4	3.5	1.9	5.8	4.4	3.5	1.9
32	5.8	4.4	3.5	1.9	5.8	4.4	3.5	1.9
36	5.8	4.4	3.5	1.9	5.8	4.4	4.1	2.7
40	5.8	4.4	4.1	2.7	5.8	4.4	4.1	2,7
44	5.8	4.4	4.1	2.7	5.8	5.5	4.5	3.0

....continued from appendix 5(b),

Time in weeks	$\ln(N_t/N_O)$				$\sum\ln(N_t/N_O)$			
	S-0	S-1	S-2	S-3	S-0	S-1	S-2	S-3
0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0
12	.26	.26	.53	.41	.27	.26	.53	.41
16	1.33	.87	.54	.27	1.59	1.13	1.04	.64
20	0	0	0	0	1.59	1.13	1.04	.64
24	.1	.32	.17	0	1.69	1.45	1.21	.64
28	0	0	0	0	1.69	1.45	1.21	.64
32	0	0	0	0	1.69	1.45	1.21	.96
36	0	0	.15	.32	1.69	1.45	1.36	.96
40	0	0	0	0	1.69	1.45	1.36	.96
44	0	.23	.08	.11	1.69	1.68	1.44	1.07

Appendix 6. Mean lengths of axillary shoots of nodal explants at 4 levels of BAP (in mm).

Time Weeks	Concentration of BAP (ppm) 2	4	8	16
4	2.4	2	2.4	1.6
8	6.7	5	3.8	2.7
12	12	10.3	8.0	4.6

Appendix 7(a). Mean axillary shoot lengths of nodal explants on six media (n=8).

Time weeks	Mean length of axillary shoots (mm)					
	S-5	S-6	S-7	S-8	S-9	S-10
4	8.1	7.4	16.0	6.0	7.1	14.2
8	8.3	10.8	20.6	9.0	12.3	21.3
12	9.3	14.0	25.3	9.5	14.5	28.3
16	11.2	15.3	31.4	11.0	17.8	30.6
20	12.9	16.3	34.9	11.2	17.9	35.6

Appendix 7(b). Shoot proliferation of nodal explants at 3 levels of sucrose on WPM media (n=8).

Time weeks	No			N_t			$\ln N_t/No$			$\sum \ln N_t/No$		
	S-5	S-6	S-7	S-5	-6	S-7	S-5	S-6	S-7	S-5	S-6	S-7
4	1	1	1	1	1	1	0	0	0	0	0	0
8	1	1	1	1	1	1	0	0	0	0	0	0
12	1	1	1	1	1	1	0	0	0	0	0	0
16	1	1	1	1	1	1	0	0	0	0	0	0
20	1	1	1	1.5	2.0	2.7	.40	.69	1.0	.40	.69	1.0
24	1.5	2.0	2.7	1.6	2.9	3.3	.06	.37	.2	.46	1.1	1.2
28	1.6	2.9	3.3	1.6	3.4	3.8	0	.16	.14	.46	1.3	1.3
32	1.6	3.4	3.8	2.1	3.4	3.8	.27	0	0	.73	1.3	1.3
36	2.1	3.4	3.8	2.7	4.0	4.2	.25	.16	.10	.98	1.4	1.4

Appendix 8(a). Mean lengths (in mm) of axillary shoots on 3 different media (n-8).

Time weeks	M&S Thi.	WPM Thi.	WPM S-1
4	5.0	4.5	5.1
8	7.1	6.9	10.9
12	8.5	9.1	15.3

Appendix 8(b). Shoot proliferation on three media (n=8).

Time weeks	No			N_t			$\ln N_t/N_o$			$\_\ln N_t/N_o$		
	WPM S-1	WPM Thi	M&S Thi	WPM S-1	WPM Thi	M&S Thi	WPM S-1	WPM Thi	M&S Thi	WPM S-1	WPM Thi	M&S Thi
4	1	1	1	1	1	1	0	0	0	0	0	0
8	1	1	1	1	1.8	1.6	0	.59	.47	0	.59	.47
12	1	1.8	1.6	3	2.0	2.1	1.1	.11	.27	1.1	.70	.74
16	3	2.0	2.1	3	2.7	2.6	0	.30	.21	1.1	1.0	.95
20	3	2.7	2.6	3	3.7	3.4	0	.31	.27	1.1	1.3	1.2
24	3	2.7	3.4	3.2	3.9	4.1	.06	.05	.18	1.2	1.4	1.4
28	3.2	3.9	4.1	3.6	4.9	-	.11	.23	.05	1.31	1.6	-

Appendix 9(a). Shoot proliferation of nodal explants on three media (n=8).

Time weeks	No			N_t			$\ln N_t/N_o$			$\_\ln N_t/N_o$		
	S-1	Thi 0.1	Thi .002	S-1	Thi 0.1	Thi .002	S-1	Thi 0.1	Thi .002	S-1	Thi 0.1	ThiS .002
4	1	1	1	1	1	1	0	0	0	0	0	0
8	1	1	1	1	1.6	1.9	0	.47	.64	0	.47	.64
12	1	1.6	1.9	1	1.8	2.8	0	.10	.41	0	.57	1.05
16	2.2	1.8	2.8	2.2	1.8	2.8	.79	0	0	.79	.57	1.05
20	2.2	1.8	2.8	2.2	2.6	3.5	0	.41	.22	.79	.98	1.27
24	2.2	2.6	3.5	3.0	3.6	3.6	.31	.34	.03	1.1	1.3	1.30
28	3.0	3.6	3.6	3.0	4.0	4.1	0	.10	.10	1.1	1.4	1.40
32	3.0	4.0	4.1	3.2	5.0	6.1	.07	.26	.41	1.2	1.7	1.81
36	3.2	5.0	6.1	3.2	5.5	8.0	0	.10	.26	1.2	1.8	2.07

Appendix 9(b). Shoot Proliferation of Nodal Explants on thidiazuron at 0.002 ppm Medium.

Time weeks	No	Nt	lnNt/N0	Σ lnNt/No
4	1	1	0	0
8	1	2.7	1	1
16	2.7	2.7	0	1
20	2.7	3.6	.3	1.3
24	3.6	3.96	.1	1.4
28	3.96	5.86	.39	1.79
32	5.86	5.86	0	1.79
36	5.86	7.97	.31	2.1
40	7.97	7.97	0	2.1
44	7.97	9.57	.18	2.28
48	9.5	12.94	.3	2.58
52	12.94	15.4	.17	2.75
56	15.4	16.97	.05	3.69
60	16.97	28.9	.51	4.2
64	28.9	28.9	0	4.2
68	28.9	33.7	.15	4.35

Appendix 9(c). Shoot proliferation on thidiazuron media (n=10).

Thidiazu: (ppm)	Time weeks	No	N_t	$\Sigma lnN_t/No$
0	4	1	1	0
	8	1	1.1	.1
	12	1.1	1.4	.34
0.002	4	1	2.5	.91
	8	2.5	3.38	1.21
	12	3.38	4.67	1.53
0.02	4	1	4	1.39
	8	4	9	2.2
	12	9	16.25	2.79
0.2	4	1	2.38	.86
	8	2.38	4.8	1.58
	12	4.8	8.6	2.17

Appendix 10. Normal growth pattern of a free growing ruuber shoot (mean length in cm).

Weeks	1	2	3	4	5	6	7	8	9
length	1.4	11	18.3	22.8	24.8	24.8	24.8	24.8	24.8

....continued,

Weeks	10	11	12	13	14	15	16	17	18
length	31.8	35.6	39.3	40.5	41.4	52.7	53.2	57.2	57.5

Appendix 11(a). Mean lengths of axillary shoots harvested from three stages of growth.

Time weeks	mean length (mm)		
	I	II	III
4	3.5	7.5	8.9
8	4.1	10.2	9.7
12	6.2	12.1	20.0
16	6.2	13.5	21.7

Appendix 11(b). Number of propagules produced per explant by the nodes of three stages of growth (n=8-16).

Time weeks	Growth stage		
	I	II	III
4	1	1	1
8	1	1	1
12	1	1	1
16	1	1	1
20	1	1	2.6
24	1.5	1.8	2.85
28	2.5	2.7	3.5
32	3.1	3.2	4.85

Appendix 12(a). Mean lengths of axillary shoots of nodes numbered from o to 7 according to their position (n=11).

weeks	0	1	2	3	4	5	6	7
4	1	1.8	4.0	4.1	6	5.2	7.4	2.0
8	2	5.6	8.1	12.4	12.1	13.8	16.6	4.5
12	3	10	18.2	19.5	24.3	29.5	21.0	5.6
16	3	10.1	18.2	19.7	24.5	32.5	21.6	8.0

Appendix 12(b). Number of propagules produced by the nodes numbered from 0 to 7, at the end of 16 weeks (n=11).

node no.	0	1	2	3	4	5	6	7
no.of prop.	1	2.3	2.7	3.0	3.5	4.6	3.9	2.1

Appendix 13. Comparison of axillary shoot growth of secondary nodal explants derived from rooted and non rooted cultures (n=15).

	Time weeks	nodes from rooted shoots	nodes from non rooted shoots
Percentage axillary bud break	4	100	100
	8	100	100
Mean axillary shoot length (mm)	4	5.9	1.34
	8	11.2	5.0
Leaf growth	4	++++++++	+++
		++++++++	+++
	8		

Appendix 14. Percentage root formation on seed embryo derived shoot tip explants on six different media (n=8).

weeks	RM 1	RM 2	RM 3	RM 4	RM 5	RM 6
4	25	37.5	37.5	50	25	25
8	25	50	37.5	75	37.5	37.5
12	25	50	37.5	87.5	37.5	50
16	25	50	37.5	87.5	37.5	50

Appendix 15. Percentage of clean cultures and cultures showing phenolic browning after sterilized with $HgCl_2$ (n=15).

	Treatment number			
	1	2	3	4
Percentage of clean cultures	50	60	60	80
Percentage of cultures with browning	5	10	5	10

Appendix 16. Percentage axillary bud break and mean lengths of 'active' and 'dormant' nodes (n=10).

	Node type	medium	Time (weeks) 4	8	12	16
mean lengths of axillary shoots (mm)	active nodes	S-0	1.2	2.0	3.0	4.5
		S-1	1.7	3.6	6.0	9.0
		S-2	1.5	3.7	8.4	17.5
		S-3	1.2	3.1	5.5	6.0
	dormant nodes	S-0	0.8	2.0	2.1	2.1*
		S-1	2.0	2.5*	2.5*	2.5*
		S-2	3.0	3.1*	3.1*	3.1*
		S-3	0.8	1.1*	1.2*	1.3*
percentage axillary bud break	active nodes	S-0	91%	91%	100%	100%
		S-1	90%	90%	100%	100%
		S-2	100%	100%	100%	100%
		S-3	100%	100%	100%	100%
	dormant nodes	S-0	50%	50%	50%	50%*
		S-1	33%	33%*	33%*	33%*
		S-2	50%	50%*	50%*	50%*
		S-3	60%	60%*	60%*	60%*

Appendix 17. Mean lengths of axillary shoots of nodal explants labelled from 0 to 5 according to their position.

Node type	Time (weeks) 4	8	12	16
0	0	0	1	1
1	2.2	2.4	6.9	7.5
2	4.4	8.5	18.2	21.2
3	3 *	3 *	3.8	11 *
4	2.5 *	2.5 *	4 *	6.6 *
5	2 *	4 *	4 *	4 *

* - Means were based on less than 5 replicates.

Appendix 18. Mean lengths of axillary shoots of nodal explants numbered from 0 to 6 (n=9).

Node number	Time (weeks) 4	8	12	16
0	0	0	0	1
1	0	1	1	1.4
2	1	5.5	6	6
3	1	7.5	8.5	10.5
4	3.5	16	16	18.5
5	2.1	13.7	13.9	14.5
6	2.1	9	9	10

Appendix 19. Mean lengths of axillary shoots of 'active' and 'dormant' nodes on M&S and WPM media (n=15).

Node type	Time	M&S	WPM
'active' nodes	4	1.7	2.0
	8	4.7	5.4
	12	8.0	10.9
'dormant' nodes	4	1.6	1.0
	8	1.9	1.3
	12	2.9*	1.9*

* - Means were based on less than 5 replicates.

Appendix 20. The mean lengths of axillary shoots on basic media at 3 levels of sucrose (n=10).

	Medium					
Time	S-5	S-6	S-7	S-8	S-9	S-10
4	2.2	2.9	3.3	1.2	3.6	4.5
8	2.9	3.8	3.6	2.2	5.8	6.0
12	2.9	4.2	4.2	2.9	6.0	6.5
16	3.5	6.9	5.9	4.0	6.5	10.0
20	4.6	8.0	12.8	4.4	7.0	10.9

Appendix 21. Mean lengths of axillary shoots on three media (n=10).

	Medium		
Time weeks	M&S Thidia.	WPM Thidia.	WPM S-2
4	3.0	2.7	2.1
8	3.0	3.9	3.0
12	4.3	5.0	5.3
16	5.4	5.8	5.2

Appendix 22. Shoot proliferation on three media (n=10).

Time	N_o			N_t		
	M&S Dropp	WPM Dropp	WPM S-2	M&S Dropp	WPM Dropp	M&S S-2
4	1	1	1	1	1	1
8	1	1	1	1	1	1
12	1	1	1	1	1	1
16	1	1	1	1.3	1.5	1
20	1.3	1.5	1	1.3	1.6	1
24	1.3	1.6	1	1.5	2.4	1.6
28	1.5	2.4	1.6	3.0	3.5	1.6
32	3.0	3.5	1.6	3.2	3.8	1.7

....continued from Appendix 22.,

Time	ln N_t/N_o			Σln N_t/N_o		
	M&S Dropp	WPM Dropp	WPM S-2	M&S Dropp	WPM Dropp	M&S S-2
4	0	0	0	0	0	0
8	0	0	0	0	0	0
12	0	0	0	0	0	0
16	.26	.4	0	.26	.4	0
20	0	.06	0	.26	.46	0
24	.14	.41	.47	.40	.87	.47
28	.69	.37	0	1.09	1.24	.47
32	.06	.08	.06	1.15	2.32	.53

Appendix 23. Mean lengths of axillary shoots of nodal explants on six levels of sucrose (n=10).

	Sucrose level					
Time	0%	2%	4%	6%	8%	10%
4	1.3	2.8	4.8	6.0	5.8	5.9
8	1.5	4.8	5.8	10.0	10.4	10.5
12	-	5.2	7.9	10.0	10.9	13.0
16	-	5.5	8.0	10.5	11.0	13.0

Appendix 24. The mean lengths of axillary shoots grown on four levels of BAP (n=12).

	Concentration of BAP (ppm)			
Time	2	4	8	16
4	4.7	3.4	2.9	1.5
8	5.2	8.6	8.8	4.6
12	5.5	9.0	9.0	5.0
16	5.6	9.0	9.0	5.0

Appendix 25. The mean lengths of axillary shoots grown on four levels of BAP (n=12).

Medium

Time	S-0	S-1	S-2	S-3
4	1.2	1.7	1.5	1.2
8	2.0	3.6	3.7	3.1
12	3.0	6.0	8.4	5.5
16	4.5	9.0	17.5	6.0

Appendix 26. The mean lengths of axillary shoots grown on four levels of 2iP (n=11).

Concentration of 2iP (ppm)

Time	0	2	4	8	16
4	2.1	2.3	2.8	1.5	2.1
8	4.3	7.7	7.7	4.5	4.3
12	4.4	8.5	8.9	4.8	4.4
16	4.5	8.6	8.9	4.9	4.4

Appendix 27. Mean lengths of axillary shoots of nodal explants of three soaking treatments (n=14).

Treatment number

Time	1	2	3
4	3.5	1.1	3.7
8	3.8	1.3	4.8
12	10.5	9.2	10.0
16	11.5*	9.4	10.9
20	11.9*	11.8	17.5

* - Means were based on less than 5 replicates.

Appendix 28. Radioactivity recovered in each fraction; three different extraction times (3X 8 hours, 3X3 hours and 3X1 hour).

		3X8hour extr.	3X3hour extr.	3X1hour extr.
Preincubation control		$1.1x10^7$	$7.5x10^6$	$5.4x10^6$
After incubation		$2.2x10^6$	$2.5x10^6$	$2.6x10^6$
Therefore uptake		$8.5x10^6$	$5.0x10^6$	$2.8x10^6$
Loss to enzyme supernatant		$5.9x10^5$	$1.2x10^6$	$8.9x10^5$
Recovery of extractions ;				
Water:	Extraction 1	$3.8x10^5$	$3.2x10^5$	$9.1x10^4$
	Extraction 2	$4.6x10^5$	$3.7x10^5$	$9.1x10^4$
	Extraction 3	$4.8x10^4$	$3.7x10^5$	$9.1x10^4$
Acetone:	Extraction 1	$1.25x10^6$	$1.2x10^6$	$8.8x10^5$
	Extraction 2	$1.46x10^5$	$1.2x10^6$	$8.8x10^5$
	Extraction 3	$1.46x10^3$	$1.2x10^6$	$8.8x10^5$
Pet ether	Extraction 1	$9.0x10^3$	$7.3x10^3$	$1.4x10^4$
	Extraction 2	$9.5x10^3$	$7.2x10^3$	$1.4x10^4$
	Extraction 3	$9.6x10^3$	$7.4x10^3$	$1.4x10^4$

Appendix 29. Radioactivity recovered in water, acetone and petroleum ether with two types of thimbles (DPM/inc.).

Solvent	Extraction	Hand made thimbles	Proprio: thimbles
Water:	Extraction 1	$1.8x10^5$	$4.9x10^5$
	Extraction 2	$4.5x10^5$	$4.9x10^5$
	Extraction 3	$8.4x10^4$	$4.9x10^5$
Acetone:	Extraction 1	$1.3x10^6$	$2.1x10^6$
	Extraction 2	$2.6x10^5$	$2.1x10^6$
	Extraction 3	$4.1x10^3$	$2.1x10^6$
Pet ether	Extraction 1	$2.8x10^3$	$1.2x10^4$
	Extraction 2	$9.3x10^3$	$1.2x10^4$
	Extraction3	$1.5x10^4$	$1.2x10^4$

Appendix 30. Comparison of Incorporation of ^{14}C-acetate and ^{14}-mevalonate into rubber stem slices of clones RRIC 110 and RRIC 121.

Precursor	Clone	Plant identification no.	Recovery as % of uptake
Acetate	RRIC 110	59	3.8
	RRIC 121	93 ,97	5.2
Mevalonate	RRIC 110	59	3.0
	RRIC 121	93 , 97	4.8

Appendix 31. The relationship between initial ^{14}C-acetate concentration and the uptake (n=3).

Initial concentration	Uptake
0	0
3×10^6	2.8×10^6
5.4×10^6	5.3×10^6
6.7×10^6	6.5×10^6

Appendix 32. Day variation of the plants on the incorporation of ^{14}C-acetate into rubber (n=3).

	Day I	Day II	Day III
Preincubation control	3.02×10^6	5.42×10^6	6.65×10^6
After incubation	2.35×10^5	9.36×10^6	1.20×10^4
Therefore uptake	92%	98%	98%
Recovery of pet ether fraction	1.39×10^5	3.06×10^5	3.50×10^5
Recovery as % of uptake	5%	5.7%	5.4%

Appendix 33. Comparison of young and mature shoots of clone RRIC 117 for their ability to incorporate ^{14}C-acetate into rubber (n=3).

	Young	Mature
Preincubation control	3.51×10^6	3.53×10^6
After incubation	2.71×10^5	3.66×10^5
Therefore uptake	92%	89%
Recovery in pet ether	6.80×10^4	1.11×10^5
Recovery as % of uptake	2.1%	3.5%

Appendix 34. Variation between individuals of clone RRIC 100 for their ability to incorporate ^{14}C-acetate into rubber.

Plant ientifica -tion no.	Pre incubation control	After incubation	Therefore uptake	Recovery in pet ether	Recovery as % of uptake
23	7.24×10^6	1.62×10^5	98%	2.13×10^5	3.0
24	3.03×10^6	2.31×10^5	92%	1.56×10^5	5.6
25	5.46×10^6	1.17×10^5	98%	3.13×10^4	0.6
26	5.42×10^6	9.99×10^4	98%	9.2×10^4	1.7
28	5.37×10^6	1.47×10^5	97%	8.49×10^4	1.6
31	7.5×10^6	1.72×10^5	98%	6.72×10^5	9.2
32	7.24×10^6	1.34×10^5	98%	6.09×10^5	8.6
35	5.53×10^6	1.4×10^5	97%	4.6×10^5	8.5

Appendix 34(b). Dry matter contents of the individuals of clone RRIC 100 (n=3).

Plant identification number	Percentage dry matter content
23	18.5
24	29.5
25	21.5
26	26.6
28	24.3
31	23.1
32	28.3
35	20.8

Appendix 35. Comparison of clones PB 86, RRIC 100, RRIC 110 RRIC 117 and RRIC 121 using the rubber producing potential.

Clone	PB 86	RRIC 100	RRIC 110	RRIC 117	RRIC 121
Preincubation control	$5.7x10^6$	$5.4x10^6$	$5.7x10^6$	$5.6x10^6$	$5.6x10^6$
After incubation	$7.1x10^4$	$1.5x10^5$	$9.3x10^4$	$1.6x10^5$	$1.3x10^5$
Therefore uptake	98%	97%	98%	89%	98%
Recovery in pet ether	$2.7x10^5$	$2.4x10^5$	$2.2x10^5$	$1.8x10^5$	$2.7x10^5$
Recovery as % of uptake	4.8	4.7	3.9	3.4	5.0

Appendix 36. Annual mean yields of the five clones (data obtained from the Rubber Research Institute of Sri Lanka).

clone	mean yield(Kg/ha/year)
PB 86	1363+16
RRIC 100	1860+289
RRIC 110	2258+166
RRIC 117	1493+108
RRIC 121	2694+375

Appendix 37. Annual yields of clones RRIC 121 and RRIC 110 for over seven years (data from RRISL).

Clone	Tapping year							
	1	2	3	4	5	6	7	8
RIC 121	1065	1743	2081	2193	3780	3312	3420	3960
RIC 110	1715	2081	1968	2081	3168	2736	2304	2016